TWO PHOTOMETRIC METHODS FOR THE DETERMINATION OF CHROMIUM IN BIOLOGICAL MATERIALS

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TWO PHOTOMETRIC METHODS FOR THE DETERMINATION

OF CHROMIUM IN BIOLOGICAL MATERIALS

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TABLE OF CONTENTS

TABLE OF CONTENTS (Concluded)

LIST OF TABLES

LIST OF ILLUSTRATIONS

SUMMARY

Chromium is a trace metal of great physiological and nutritional interest. It is essential to certain normal metabolic processes and in recent years the measurement of chromium levels has become an important diagnostic tool.

Two photometric methods have been developed during the course of this investigation that allow chromium determinations in biological materials.

The first method uses diphenylcarbazide as the colorimetric reagent. Previous photometric methods for chromium have used diphenylcarbazide but have required large amounts of sample material. This fact has limited the use of diphenylcarbazide in diagnostic testing. But the method developed during this study requires only 200 milligrams (or 200 microliters depending on the physical state of the material) of sample material. This large reduction in the amount of material needed is possible through the use of a long-path microcell. The increased path length without a proportional increase in cell volume makes it possible to determine extremely small concentrations of chromium in relatively small amounts of biological materials.

Unfortunately, it is not possible to determine the chromium directly in the untreated sample. The organic material must be destroyed before the determination can be performed. This necessitates a digestion step. It was found that for chromium determinations, a mixture of concentrated sulfuric, nitric, and perchloric acids in a volume ratio of 15:3:2

was best for the digestion. Also, before diphenylcarbazide can be used the chromium must be oxidized to the hexavalent state. This is accomplished with a permanganate oxidation.

The diphenylcarbazide method was used to determine the chromium concentration of three different serum pools. These three pools were also analyzed for chromium by a flameless atomic absorption method and the results compared with those obtained by the diphenylcarbazide method. There was no significant difference between the results on the 95 percent confidence level.

The second method developed is based on a different photometric approach. The o-tolidine method relies on the oxidizing power of the chromium(VI). o-Tolidine is oxidized by chromium(VI) to the corresponding intensely yellow quinonediimine. Strictly stoichiometric oxidation is achieved with manganese(III) pyrophosphate as a mediator. The quinonediimine has an apparent molar absorptivity based on the chromium concentration of 45,300 1/mole-cm which is approximately three times the apparent absorptivity of the chromium(VI)-diphenylcarbazide.

The serum pools used in the evaluation of the diphenylcarbazide method were analyzed for chromium by the o-tolidine method. This method gives slightly yet consistently higher results. Tests have indicated vanadium as the probable cause of this effect. Therefore until more is known about the vanadium interference caution is suggested when evaluating chromium levels determined by the o-tolidine method.

INTRODUCTORY REMARKS

According to present knowledge, the human body contains some thirty-seven elements of which twenty-six are metallic in nature; many are present in only trace amounts. The importance of several of these metals in mediating biochemical processes has promoted investigations of their physiological values and the effect of alterations in these values in physiological states.

One of these metals is chromium. It is essential to certain normal metabolic processes and in recent years the measurement of chromium levels has become an important diagnostic tool; it is also of interest because of toxicological aspects. In man, normal blood chromium concentrations are at the level of 10 to 50 nanograms per milliliter. To better establish the relationship between chromium and certain body functions, such as glucose metabolism, a method to analyze for chromium is needed. Evidence that chromium deficiency is a factor related to disturbances of glucose metabolism (1) points out the need for an even more sensitive method, namely one that permits analysis of chromium at levels down to two nanograms per milliliter.

A variety of methods for the detection and measurement of chromium in biological materials has been described, including spectrophotometry, arc emission spectrography, spark source mass spectrography, atomic absorption spectrometry, both flame and flameless modes, coulometry, polarography, neutron activation analysis, and gas-liquid chromatography. All of these methods have their good points and all, unfortunately, have their

bad points. Therefore, the decision of which is best in a given situation becomes very difficult and many factors must be weighed.

This investigation has sought to develop methods for the determination of chromium in biological materials that do not have the drawbacks of the existing methods, while still maintaining the favorable aspects.

At present, atomic absorption using a graphite furnace, neutron activation analysis, and gas-liquid chromatography are the most accepted methods of analysis. Each of these methods offers the sensitivity necessary for the determination of chromium in biological materials and need very small samples. But they all require a considerable investment in instrumentation, and unfortunately, do not lend themselves very well to automation, using existing clinical instrumentation. If the determination of chromium is to become a frequently used diagnostic tool, then the ability to automate a method will become very important.

This investigation has developed two photometric methods which have the necessary sensitivity, need about the same small amount of sample as atomic absorption, neutron activation, or gas-liquid chromatography, but do not require the high initial investment. The new methods should be readily adaptable to automation because all of the steps involved have already been used in automated analysis.

CHAPTER I

THE ROLE OF CHROMIUM IN BIOLOGICAL SYSTEMS

Evidence accumulated during the past three decades has created a considerable degree of interest in the biological action of chromium. Chromium(VI) has been considered a toxic metal for many years and toxicity studies involving subjects exposed to industrial dichromates have been reported (2-6). Some of these studies include measurements of chromium levels in tissues, blood, and urine (4-6). Chromium(III) is less toxic than chromium(VI) and evidence is accumulating that the trivalent ion plays a much more important role in normal biological metabolism than does the hexavalent. Curran (7) in 1954 showed that cholesterol and fatty acid synthesis by rat liver was enhanced by the presence of chromium(III). In addition, there is evidence that in rats chromium(III) may be an essential element for optimum utilization of glucose (8-10). Other reports have suggested that some cases of human diabetes mellitus can be improved by supplementing the diet with trace amounts of chromium-(III) (10-12).

Theories of the functional relationship of chromium(III) to glucose utilization evolved from studies on the dietary glucose tolerance factor (13-16), which is a measure of an individual's ability to use up blood glucose. Patients with mild or diet-controlled diabetes when fasting may have blood glucose levels within the normal range, but are unable to produce sufficient insulin for prompt metabolism of ingested carbohy-

drates. As a result, blood glucose rises to abnormally high levels and the return to normal is delayed; that is, the patients have a decreased tolerance factor for glucose. After chromium(III) had been shown to be effective in increasing the factor (17), continued studies have found that chromium(III) and physiological levels of insulin are required for glucose or galactose uptake into the epididymal fat tissues of chromium-deficient rats (18,19). Chromium(III) has been reported to enhance the effects of insulin, not only on glucose metabolism, but also on other systems, by facilitating the initial reaction of the hormone with the respective receptor sites on membranes (20,21).

The understanding of the function of chromium in biological systems requires a knowledge of the fundamental chemistry of chromium.

Chromium, with an atomic number of 24 and an atomic mass of 52.01, belongs to the first series of the transition elements. It is positioned in subgroup VIB of the periodic system and surrounded by three elements with known biological function: vanadium, manganese, and molybdenum. Chromium has been found to occur in every one of the oxidation states from -2 to +6, but only the 0, +2, +3, +6 are common. Divalent chromium is very unstable. This is indicated by the standard potential, $E^\circ = -0.41V$ for $Cr^{3+}\ e^- \rightleftarrows Cr^{2+}$. Because of this high reducing power, chromium(II) compounds are unlikely to occur in biological systems.

The hexavalent form is a strong oxidizing agent and is almost always linked with oxygen. It differs from the highest oxidation state of molybdenum and tungsten in that it has far less tendency to form poly-acids, and the only important ions of chromium(VI) are chromate, CrO_4^{-2}, and dichromate $Cr_2O_7^{-2}$. The dichromate is most prevalent in acid solution

and is reduced to chromium(III) according to:

$$Cr_2O_7^{-2} + 14H^+ + 6e^- \rightleftarrows 2Cr^+ + 4H_2O, \quad E^\circ = 1.33V$$

The trivalent is the most stable oxidation state. Chromium(III) has a strong tendency to form coordination compounds. The rate of ligand exchange of such compounds is very low, and therefore can be followed quite easily. Chromium(III) has a coordination number of six, with the ligands attached at the corners of an octahedran.

The chemical properties of trivalent chromium can serve as a basis to delineate some elementary functions that chromium can be expected to have in biological systems.

i) The differences in oxidation potentials of chromium(II) and chromium(III) and chromium(III) and chromium(VI) are so great that a reversible transition between two oxidation states is extremely unlikely in biological systems. Therefore, the role of chromium in biological media is different from the role of iron in the cytochrome system, which depends on the easy transition Fe(II) $\rightleftarrows$ Fe(III), or for the molybdenum in enzymes, which depends on Mo(V) $\rightleftarrows$ Mo(VI). Chromium most probably functions only in one oxidation state, namely chromium(III), and therefore is not likely to participate in biochemical oxidation-reduction reactions.

ii) Chromium(III) occurs only in one configuration, the octahedral. In this respect it differs from metals with a coordination number of four, which, depending on the nature and charge of the ligands, can function in two configurations (planar and tetrahedral).

iii) Chromium(III) is not subject to high-spin, low-spin transi-

tions that are essential in the function of iron(II) as the carrier of oxygen in hemoglobin.

iv) Chromium(III) is surpassed only by cobalt in the low rate of ligand exchange. This property would make chromium(III) complexes unsuitable as an active site of enzymes in which the catalytic activity of the metal depends on a high rate of ligand exchange. The inertness of the chromium-ligand bond would rather be compatible with a more structural function; for example, the binding of hormones to receptor sites on the stabilization of tertiary structures in proteins on nucleic acids.

The free chromium(III) hexaquo complexes do not exist at physiological pH values. In the absence of stabilizing ligands at physiological pH values, formation of polynuclear chromium hydroxo complexes of colloidial nature and of little or no biological activity would occur. Reaction of chromium(III) with ligands that successfully compete with OH^- for coordination protects against hydroxide precipitation in neutral media. Blood and other biological fluids contain a variety of compounds that can easily coordinate with chromium, and this is the basis for the solubility of chromium(III) in biological material (22-24). The interaction between chromium and adenosine triphosphate (ATP) and its derivatives has been known for twenty years (25). The reactions between chromium and low-molecular-weight substances of biological importance at physiological pH values have been extensively studied by Rollinson (26-28). One paper (28), in particular, deals with the reaction of a number of biologically important ligands of chromium. The ligands studied were glycine, leucine, lysine, methionine, pyrophosphate, and serine. The diffusion rate of chromium from each of the chelates was measured and the following order

in which the ligands maintain chromium in a diffusible form was established: pyrophosphate > serine > glycine > leucine > lysine > proline. Glucose did not influence diffusion rates at all; oleate depressed them strongly, probably through the formation of large, chromium-containing micells.

These investigations had an important bearing on the understanding of the behavior of chromium in organisms. If given orally in the form of simple salts (e.g., $CrCl_3 \cdot 6H_2O$), chromium hydroxide would start precipitating in the alkaline medium of the duodenum. But ligands are present in the intestines that chelate chromium and thus keep it in solution and make it available for absorption. The great affinity of chromium for pyrosphosphate is of special interest, since a number of pyrophosphate derivatives are of paramount biological importance. The fact that glucose does not appreciably react with chromium allows the important exclusion of one theoretically feasible mode of action (18).

Proteins

The earliest known and probably best understood reaction of chromium with proteins is the tanning process, which was developed empirically but acquired its scientific foundation with the advent of coordination chemistry. Much of the knowledge accumulated in studying tanning processes is useful for the understanding of the chromium chemistry in biological systems. Tanning transforms skin collagen into leather through cross-linking by the tanning agent of the elementary fibrils of the collagen (29). In chrome tanning, chromium reacts mainly with the free carboxyl groups of the acidic amino acids of the protein (glutamic and aspartic

acids), forming stable complexes between the sites of different chains of protein (29). Other binding sites, hydroxyl groups, peptide bonds, and amino groups, have been suggested, but probably play only a minor role, since masking these does not impair the tanning process appreciably (30). Methylation of the free carboxyl groups, on the other hand, prevents tanning completely (29). The role of chromium in the tanning process is still not completely understood at the present time due to the fact that a large number of different chromium species are generated in the process. However, the following, all of which may have a general biological significance, are unequivocally established. 1) Only chromium(III) has tanning ability; chromium(VI) acts only after reduction to the trivalent form. 2) Tanning involves the coordination sites of chromium(III). 3) Mononucleate chromium(III) complexes do not tan.

The tanning reaction is initiated by raising the pH of the solution, which causes the formation of polynucleate complexes. These act by accepting carboxyl groups of the collagen strands into their coordination sphere at the expense of previously bound water molecules. It must, however, be realized that tanning involves a total saturation of protein with chromium, a situation that does not occur in the living organism. The chromium content of leather ranges from 3.8 to 5.6% (26), whereas that of living matter, for example, muscle, is around 50 nanograms per gram or 0.000005%. Within this range only a very small fraction of the potential binding sites are occupied by chromium. It is most likely that the sites reacting at normal chromium concentrations are different from those reacting at higher concentrations.

The cross-linking by chromium and other elements of a protein in

solution has been investigated with conarachin II, isolated from groundnuts. The presence or absence of cross-linking in the protein at pH 5.6 was followed by ultracentrifugation of the reaction products. Ca(II), Zn(II), Fe(II), Hg(II), Cu(II), Mn(II), Pt(IV), Th(IV), and Ti(IV) failed to cross-link the protein at molar ratios of metal:protein from 1:2 to 30:2. Al(III), Fe(III), and Cr(III), on the other hand, did produce cross-linking, but with an important difference. Aluminum and iron reacted quite rapidly, but the metal-protein complexes were unstable and easily broken by dialysis against phosphate. The reaction of chromium proceeds more slowly, but the resulting complexes were stable and resistant to the action of phosphate. These findings indicate the formation of coordinative links through which chromium, but no other metal, binds to the protein (31). Stabilization of certain tertiary structures of proteins by physiological concentrations of chromium appears possible on the basis of these results and the properties of the chromium-ligand bond. However, there is evidence that chromium can stabilize polypeptide chains in either random-coil or spiral conformation and that it can prevent thermal transition from one form into the other (32). But when present in excessive amounts chromium, in the form of cationic or anionic complexes, precipitates proteins from solutions. The reaction is easily reversed and no denaturation occurs (33).

The interaction of proteins with chromium(III) and chromium(VI) has been investigated via dialysis and electrophoresis (34). At pH 7.35, egg protein and human plasma protein were found to strongly bind chromium(III) presented as the acetato complex, but not chromium(VI), administered as dichromate. The latter reacts with the protein at a pH of 4, with

decreasing binding as the pH was raised from 4 to 7.35. Lipemia of plasma decreased the binding of chromium(III) to the proteins, in agreement with the findings of Rollinson et al. (28) about the effect of oleate. More detailed measurements subsequently demonstrated that of the serum proteins, siderophilin bound almost all the chromium administered in physiological doses (1 μg/100 g body weight) (35). When excessive amounts of chromium were given, other protein fractions also bound chromium, at the expense of the amount in siderophilin. This distinguishes the behavior of chromium from that of calcium, copper, zinc, and cobalt, all of which were shown to bind the siderophilin in a nonspecific way (36). Since the great affinity of chromium for siderophilin was also shown after chromium was administered orally to laboratory rats, it is likely that binding to siderophilin is the mechanism by which ingested chromium is carried to the tissues. Since iron rarely occupies more than one third of the available binding sites, the small amounts of serum chromium (20-30 ng/ml) can be easily handled. The binding is firm enough to prevent nonspecific "tanning" reactions of chromium with other protein sites and yet labile enough to allow chromium to be removed from its carrier then chromium is taken up by the tissues.

Enzymes

Chromium can also interact with certain enzymes present in serum. Stimulation of oxygen consumption in a succinic-cytochrome c dehydrogenase system by chromium(III) has been reported (37). Of the metals tested (Al, Cr, Nd, La, Sm), chromium had the greatest effect, producing approximately twice the stimulation of the next effective element, aluminum, compared on

a mole-to-mole basis. However, biological significance could be attributed only to the aluminum, since only this metal was present in detectable concentrations in the enzyme preparations used.

The enzyme phosphoglucomutase, which is important in the early steps of glucose metabolism, also requires chromium for functioning (38). However, this requirement appears only when the enzyme is used in dilution. In the absence of metallic co-factors the activity drops to zero with increasing dilution, but no decline occurs over a wide range when two active metals are added to the system. The presence of Mg(II) is required for maximal activity, together with a second metal, which can be Cr(III), Fe(III), Al(III), Pb(II), UO_2(II), Ti(IV), Ba(II), La(III), Sn(II), Zn(IV), Cs(I), or Ti(I). In this system chromium(III) was found the most effective second metal at all concentrations used; the maximal activity is obtained with concentrations of 10^{-5}M. Also, chromium is the only element that maintains enzyme activity in the absence of magnesium; but there, higher concentration (3×10^{-4}M) is required. This suggests that chromium may play a key role in phosphoglucomutase activity, particularly since the effective concentrations of 50 ng/ml in the system do not differ much from naturally occurring levels.

Chromium or manganese in concentrations of 5×10^{-5}M stimulates the conversion of acetate to carbon dioxide, cholesterol, and fatty acids in rat liver (7). Injection of 250 μg of chromium per 100 g body weight into donor animals leads to the same response in the liver after one hour. The effects of equal amounts of chromium(III) and manganese(II) were very similar, but chromium was more easily antagonized by simultaneous addition of vanadium than was manganese. When administered by injection, chromium

was somewhat more effective than manganese in equimolar amounts.

The observation that the conversion of acetate into carbon dioxide and into fatty acids was also stimulated by the metals suggests additional sites or a common one that could increase utilization. One such common site has been postulated for the metabolism of acetate in epididymal fat tissue, but in these experiments the presence of glucose and insulin was required for the chromium to take effect (19).

There is evidence that the digestive enzyme trypsin contains one atom of chromium per enzyme molecule. Chromium can be removed by dialysis, indicating that it is not firmly bound. The residual activity of trypsin after dialysis is only 5% of the original, and it is restored to normal by the addition of chromium or addition of six times greater amounts of magnesium. Solutions of trypsin, dialyzed for 96 hours against 0.15 F phosphate buffer, lost more than 90% of their initial activity. Addition of chromium to the buffer solution outside of the dialysis bag in concentrations equimolar to that of the enzyme not only preserved the initial trypsin activity, but actually increased it to approximately 125% of the initial value. Fe(II), Mg(II), Al(III), and Mn(II) gave increased activity, whereas Zn(II), Co(II), and Cu(II) were ineffective. Chromium was also reported to stimulate the activity of the protein-splitting enzyme, rennin, as measured by an increased rate of milk coagulation (39).

Chromium can inhibit enzyme reactions when concentrations excessive for the particular enzyme are given. The following systems have been reported to be inhibited: bacterial urease (1-10 μg dichromate/ml) (40), thermoboplastic activity (25 μmoles chromium/200 μg thermoboplastic protein) (41), and β-glucuronidase (42). These effects are not specific for

chromium and are observed with other metals that have as great an affinity for functional groups on protein as chromium. Any amount of metal in excess of the saturation level of the specific acceptor sites will react with other sites on protein molecule. These sites may be nonessential for the function, but if they are essential, coordination of the metal will result in a depression of the function. The intact organism can usually maintain efficient control of its trace elements, not only regarding their concentrations, but also the chemical form in which they are bound. Therefore, whether depression or stimulation of the system is observed will depend on the amount of the element added (43). The nature of the interaction of chromium with the protein is clearly dependent on the concentration. For example, chromium serves as an activator of trypsin in a metal: protein ratio of 1:1, and in approximately the same ratio to insulin, chromium initiates the reaction of insulin with the receptor sites. But at 10:1 or 20:1 ratios, chromium already begins to form cross-links with conarachin II (44). Oversaturation of transferrin with chromium results in the binding of the chromium to nonspecific sites (1). It is possible that this nonspecific binding does not occur when chromium is added in the form of its "natural" complexes.

Nucleic Acids

The interaction between chromium and nucleic acids was first pointed out after the treatment of tissues with chromates and dichromates greatly reduced the amount of nucleic acids extractable with trichloroacetic acid (45). The effect was quite specific for chromates and dichromates and was not seen with the use of acids other than trichloro-

acetic. Since the tissues assumed a greenish color after the treatment with chromate and dichromate, it is probable that reduction to chromium-(III) and subsequent complex formation with nucleic acids occurs.

The relation of nucleic acids and chromium, as well as other metals, was thoroughly investigated by Wacker and Vallee (46) who found chromium concentrations from 260 to 1080 μg/ml in a beef liver extract consisting of 70% ribonucleic acid and 30% protein. This is the highest concentration of chromium ever reported in living material. In subsequent experiments, these workers measured the chromium contents of ribonucleic acids from different sources. Chromium contents varied from a high of 400 μg/ml in horse-kidney-RNA to a low of 18 μg/ml in calf-pancreas-s-RNA. The authors were unable to establish a consistent stoichiometric ratio between chromium and RNA; however, the ratio between chromium and RNA-phosphorus was remarkably constant.

The chromium-RNA complex had the highest bond stability of any of the metal-RNA complexes studied by Wacker and Vallee. Also shown was that many other metals "stabilize the ordered structure of RNA"; however, the stabilization was maximized in the case of chromium.

With regard to the chromium-RNA complex, there are three areas in which chromium can be distinguished from other metals: 1) concentration (extremely high levels in nucleoproteins); 2) stability of attachment; and 3) protection against heat-induced conformational changes in the RNA structure.

Red Blood Cells

Gray and Sterling (47) were the first to discover the great affinity of the chromate and dichromate ions for the red blood cells. Labeling of erthrocytes with chromium(VI) has found wide application as a diagnostic and research tool. When chromium(VI) is added to blood, it penetrates the erythrocyte membrane rapidly without appreciable reacting with the components of the plasma. The trivalent form, on the other hand, attaches itself firmly to the plasma proteins without migrating into the blood cells. The chromium(VI) must penetrate the red cell membrane rapidly because the transport process is in competition with the reducing capacity of plasma constituents. Once inside the cells, the chromium(VI) is reduced to chromium(III) and bound to hemoglobin, resulting in a stable tagging of the erythrocyte (47). That the intracellular chromium is trivalent is indicated by two facts: 1) chromium, once inside the red cell is unable to penetrate the cell membrane for the purpose of leaving the cell and 2) chromium(III) has a much greater affinity for a direct reaction with hemoglobin than does chromium(VI). Most of the chromium is bound to the globin portion; however, binding to heme and to a substance of low molecular weight has also been demonstrated. The chromium-containing complex with the low molecular weight substance can be isolated from chromate-treated, lysed erythrocytes by ion-exchange. This complex exhibits no more biological activity than simple, inorganic chromium compounds (1).

Glucose Metabolism

Chromium also increases the effect of insulin on the utilization of glucose carbon for fat synthesis (18) as well as for oxidation to carbon dioxide. Maximal effect of chromium on lipogenesis was obtained with labeled glucose and insulin present in the system, but the utilization of acetate-^{14}C was also stimulated by chromium, provided glucose and insulin were added to the medium (17). Small increases in the incorporation of acetate carbon into fat in chromium-supplemented tissues were insignificant whenever glucose and insulin were missing from the system. The fact that glucose uptake and the utilization of glucose for two different pathways are affected by chromium to approximately the same extent and the ineffectiveness of chromium in the absence of insulin suggests that the site of the action of chromium is near the site of the action of insulin. It is well established that the mechanism by which sugar is translocated across the cell membrane in muscle and fat tissue is responsive to insulin although the membrane is not the only site of action of the hormone (48). d-Galactose has been shown to enter the muscle cell by an insulin-dependent mechanism. Once inside the cell, the sugar is not phosphorylated and therefore not metabolized, and it accumulates until its concentration in the cell water equals that in the extracellular liquid. The rate at which equilibrium is reached is increased by insulin. This system was used to determine the effect of insulin and chromium on membrane translocation of sugars without interference from intracellular metabolism (19). Concentrations of 10-100 ng chromium per 100 mg of epididymal fat tissue significantly increased the rate of entry of galactose over the rate effected by insulin alone. Chromium was ineffective in

the absence of insulin, but at the optimal concentration of insulin the rate was observed to increase. The best effect was produced by 10 ng of chromium for approximately 100 mg of tissue containing the optimal concentration of insulin; higher and lower doses were less effective.

The observed effect of chromium on the rate of cell entry of d-galactose established the fact that the insulin-responsive cell membrane is one site of action of chromium. Even though an action at this site can account for this effect of chromium and insulin on the glucose metabolism, it is evident that both chromium and insulin must have additional sites of action.

The mode of interaction between chromium and insulin is not clear. There are at least five possibilities to consider (1). i) Chromium forms a complex with the insulin in the pancreas or in the blood. The element may serve to maintain the polypeptide chains in an optimal tertiary configuration. ii) Chromium acts as an inhibitor of tissue insulinase. iii) Chromium strengthens the initial binding of insulin to tissue. iv) Chromium acts as a co-factor of a membrane-carrier structure involved in glucose transport. v) Chromium acts as a catalyst in the initial reaction between insulin and a specific membrane receptor site. All five of these possibilities are being explored at the present time.

Chromium Transport

Up until now the emphasis has been on the effect of chromium in biological systems once the chromium is in the system. The question still remains as to how the chromium gets into these systems. Chromium(III) is poorly absorbed from the gastrointestinal tract. However, some chromium

is absorbed through the intestinal walls. The site of chromium absorption is unknown, as is the mechanism by which the chromium is carried across the intestinal wall. There is indirect evidence that the chemical state of chromium in the intestinal tract determines the extent of absorption (49). Unless protected by complexation, chromium, in the alkaline medium of the intestinal contents, would precipitate in the form of large, insoluble entities. Therefore, it is possible that the degree of absorption depends on the efficiency with which suitable ligands protect against precipitation.

Once absorbed, chromium(III) appears in the plasma protein fractions in a dose-dependent distribution. Small, physiological amounts are bound almost entirely to siderophilin, the iron-binding protein (36). With abnormally large concentrations of chromium, binding also occurs on other proteins, once the siderophilin has been saturated. The subsequent uptake by the tissues appears to be dependent on the chemical state in which the chromium was administered (50).

Excess circulating chromium is excreted with urine and feces. Urinary excretion is the major route, accounting for at least 80% of injected chromium in experiments conducted with rats, but elimination via the intestines also plays a role.

Toxicology

Some mention of the toxicological effects of chromium in biological systems should be made. Toxic effects are part of a dose-response relation not only of chromium but of every substance in nature. Toxicity of a substance can be related to the ratio of beneficial or essential doses

to those that are deleterious; this relation is more meaningful than absolute toxic levels. Also, the toxicity of a substance must be studied with respect to two completely different time and dose scales. First, a large dose of the substance received within a short period and second, the same dose given in small increments over a very long period. The toxicity of chromium when a large dose is received within a short period of time has been recognized for many years. But, studies into long range toxicity of chromium are just now being started, because until now methods sensitive enough to determine trace amounts of chromium did not exist. The results of these studies will not be available for some time. Until then the toxicological effects of chromium must be explained with what is known about the chemistry of chromium in biological systems.

Chromium(VI) penetrates the cell membranes quite readily and is a strong oxidizing agent; these two facts are the basis for its irritating effects and a toxicity greater than that of chromium(III). Chromium(VI) is irritating when locally applied in high concentrations. The increased incidence among chromate workers of acute and chronic diseases of the respiratory system is well known (52). Among these ailments are: ulcers and perforations of the nasal septum, rhinitis, sinusitis, laryngitis, asthma, acute chemical pneumonitis, and bronchiogenic carcinoma.

The toxicity of chromium(III) appears to be restricted to parenteral administration. The minimal lethal dose for humans has been given as 2.29 g per kg of body weight for chromic chloride (53). These data point out the very low toxicity of chromium(III).

The consistent occurrence of the element in biological matter does not prove its essentiality. A specific affinity for certain tissues or

substances is suggestive of a biological role, but does not constitute proof. It is difficult to distinguish between trapping of excessive amounts and accumulation at functional sites. The facts that chromium is present in relatively high concentrations in the newborn organism, that it has a predilection for proliferating tissues, that it accumulates in very high concentrations in certain areas of the brain and in nucleic acids, and that it undergoes significant concentration changes in serum when glucose needs to be metabolized, all indicate a biological role of chromium.

In evaluating the role of chromium in animal and human nutrition, the deficiency criterion is of great importance. Low-chromium states exist in man. Most cases are mild; therefore, the degree of impairment and the magnitude of effects of chromium resupplementation are not dramatic in most instances.

The determination of the nutritional state with regard to chromium in population groups is an important goal for nutritional research. Although the time-consuming therapeutic trail is as yet the most reliable tool to detect deficiency states, it can be expected that when simple analytical methods become available great strides will result.

CHAPTER II

SURVEY OF EXISTING INSTRUMENTAL METHODS

As has been mentioned before, there have been three techniques recognized as "standard" methods for the determination of chromium in biological material: neutron activation, gas chromatography, and atomic absorption. All three possess the high sensitivity necessary, with interferences at a minimum.

Neutron activation analysis is based on the fact that many elements become radioactive when bombarded with thermal neutrons, that is, neutrons with a kinetic energy less than 0.2 eV.

A thermal neutron can be captured by an atomic nucleus to give a heavier nucleus with the same positive charge, that is, an isotope of the same element is formed. Such nuclei in many cases are unstable and spontaneously disintegrate with emission of a particle or gamma-ray or both. The resulting activity can provide both qualitative and quantitative data. Active isotopes of the various elements differ widely in half-life, and in many instances can be identified by the half-life, or other pertinent information, such as the gamma-ray energy spectrum.

When the intensity of the radioactivity of a sample is plotted against time, a so-called decay curve is obtained. This curve is generally complex in nature, being the sum of the activities of all the active elements present. The more active elements present the more complex the curve. The half-life of the longest-lived components can be determined

from the latter portions of the curve after most transient elements have virtually vanished. The activity due to this element can then be subtracted point by point ("stripped away") from the reading resulting from shorter-lived elements. Then the next longest-lived substances can similarly be identified and stripped away, the next and so on. As can be seen, this could result in a counting period of days, sometimes weeks depending on the half-lives of the elements produced. Therefore, in order to save time, it is preferred to obtain the desired results by running a particle or gamma-ray energy level spectrum rather than plotting a decay curve. The energies of the particles or gamma rays emitted during the decay of active elements vary depending upon the elements present. A multichannel analyzer allows the measurement of a wide number of discrete energy levels depending on the number of channels in the analyzer.

Neutron activation offers possibilities for both qualitative and quantitative methods of analysis. The value of the emitted energy level yields qualitative data while the relative intensity of that particular energy level is a quantitative measure of a particular element.

The sensitivity of analysis by means of neutron activation depends on the intensity of the activating neutron beam, on the ability of the sought-for element to capture neutrons, that is, the neutron-capture cross section, and on the half-life of the induced activity. The governing relation is:

$$A = N\sigma\varphi \left[1 - \exp\left(\frac{-0.693t}{T_{\frac{1}{2}}}\right)\right]$$

where A is the induced activity at the end of the period of irradiation,

in disintegrations per second; N, the number of atoms present of the isotope being activated; σ, the neutron-capture cross section in square centimeters; φ, the flux in neutrons per square centimeter per second; t, the irradiation time; and $T_{\frac{1}{2}}$, the half-life of the product. Quantitative analysis is rarely based on calculations from this equation, as sufficiently reliable data are seldom available for σ, φ, and $T_{\frac{1}{2}}$; as a further complication, φ may not be homogeneous and may vary with time. For practical purposes, standard samples are irradiated simultaneously with the unknowns, and the evaluation carried out by comparison.

If a powerful neutron source such as a nuclear reactor, $\varphi = 10^{12}$, is available for activation, in favorable cases, as little as 10^{-10} g of an element can be detected (54). For less powerful neutron sources, such as the combination of ^{124}Sb and Be($\varphi = 10^{4}$), this technique is limited to those elements which have particularly favorable nuclear properties. In the case of chromium in biological material, a nuclear reactor is required for activation analysis.

All accepted methods for the determination of chromium in biological material by activation analysis, require the sample to be digested. After digestion, the sample is then irradiated in a reactor for a period ranging from 3 to 8 hours depending on the sophistication of the analyzer used. The intensity of the gamma-ray emission of the irradiated sample at the energy level of 320 keV is monitored. This intensity is proportional to the amount of chromium present. Aliquots of the sample can be spiked before digestion with a known amount of chromium and the activity of the spiked sample be used to determine the actual amount of chromium in the sample before the standard addition.

The complete procedure for the neutron activation analysis of biological materials for chromium can be found in the work of Piper and Goles (55).

At first glance methods using neutron activation appear very promising for chromium determination but these methods have two very imposing problems as far as routine analysis goes. First, the equipment needed is beyond the reach of an ordinary clinical lab. Second, although the time required to measure the intensity of a discrete energy level is generally small, because of the large amount of alkali metals found in many biological materials, a "cooling down" period for irradiated samples is required before the intensity at 320 keV can be measured. The period may range from several hours to several weeks depending on the composition of the material being analyzed. Thus, neutron activation analysis as a means of rapid determination of chromium in biological materials is eliminated.

Several methods exist which use gas chromatography for the determination of chromium in biological material (56-58). Some of these methods do not even require digestion of the sample. All rely on the formation of chromium β-diketone chelates (57). The chelates investigated thus far have been derived from acetylacetone (59), trifluoroacetylacetone (60), hexafluoroacetylacetone (61), and heptafluoracetylacetone (62). Many workers studying the fluoro-β-diketones have employed the electron capture detector because of its exceptional sensitivity to halogenated organic compounds. Nanogram amounts of chromium can be determined using electron capture detectors.

These detectors are based on the fact that gases normally are not electrical conductors, but can be ionized and thus rendered conductive.

In general, the resistance of a stream of an ionized gas is affected by its composition and this fact can serve as the basis for detection. There are several ways that a gas can be ionized. The most common methods are flame ionization and electron capture. But no matter what ionization process is used, all ionization detectors are similar in that the ionized gas stream passes between a pair of electrodes that have a voltage imposed. The detector response takes the form of variations in current flow.

In an electron capture detector ionization is effected by the presence of a short-range β-emitting source such as ^{63}Ni. In operation, the β source produces a steady stream of electrons that are collected by the electrodes, providing a steady state current output of the detector. Introduction of a compound having a significant cross section for capture of thermal electrons results in the formation of ions having very much lower mobilities than the electrons. As a result, the current output of the detector decreases in the presence of a compound to which it is sensitive.

The response of an electron capture detector is not linear. It can be described by an equation analogous to that of Lambert-Beer's law:

$$I_b = I_a e^{KC}$$

where I_a is the steady state current observed with the carrier gas only, I_b is current in the presence of gas and a sample component, K is a constant describing the detector performance, and C is the concentration of the compound responsible for electron capture.

The value of this detector lies in its ability to show very high response for a relatively small number of compounds having large electron-

capture cross sections, while not responding to a great many other compounds. It is therefore, capable of detecting very small concentrations of those compounds to which it is sensitive, especially halogenated organics, and polyaromatics, in the presence of large amounts of other compounds.

In the gas chromatographic methods for chromium, being used at present, there are two ways of forming the chelates in the organic matrix. The first approach involves digestion of the biological material, followed by chelation. The other approach uses a solvent extraction procedure. The chelating agent is added to the sample. The mixture is heated in a closed container. This mixture is then extracted with an organic solvent. With a fluoro-β-diketonate excellent sensitivity from electron-capture detection can be realized. Also as an added feature of the electron capture detectors, their insensitivity to all but a few organic solvents avoids many problems associated with a solvent extraction step.

Regardless of the technique used to form the chromium β-diketonates, separation from excess reagent and other diketonates is achieved by the use of columns that contain silicone gums as the liquid phase.

Burgett's method (63) is representative of most gas chromatographic methods now being used for the determination of chromium in biological materials.

Gas chromatographic methods do offer some improvement over neutron activation in the amount of equipment required, but if large numbers of samples are to be processed, gas-liquid chromatography is not the method of choice.

Atomic absorption spectrophotometry is another technique that is

used to determine chromium in biological material. Tremendous amounts of work have been done on the development of atomic absorption methods for trace metals in biological systems. The reduction of interferences is among the reasons for much of the work.

Williams et al. (64) studied the problems involved in the determination of chromium in feces. They used a phosphoric acid, manganese sulfate and potassium bromate mixture for digestion and an acetylene-air flame, Operation was over a chromium range of 0.8 to 70 μg/ml, using aqueous solutions. Delaughter (65) determined ng/ml concentrations of chromium in biological materials by atomic absorption. After digestion of the sample, chromium is chelated with diphenylthiocarbazone and extracted into methyl isobutyl ketone. The method is extremely sensitive, with the limit of detection at one ng/ml. However, at least 800 g of sample is required. With that amount needed for one determination, the use of atomic absorption, in the flame mode, would not meet the requirements for a method to be used on a routine basis.

The determination of trace chromium in biological material by flame atomic absorption has been, and still is, fraught with the complexity of the sample, sample pretreatment, standard preparation, background effects, and in some instances the lack of sensitivity. These problems necessitated the development of new atomizing techniques to achieve the required sensitivity for microliter samples having a minimum amount of pre-treatment. This has been approached by the introduction of the graphite furnace, the heated graphite cell, the carbon rod atomizer, the graphite cup, the gold plated graphite cup, and the tantalum strip.

Although application of the above atomizing techniques resulted in

a 1000-fold increase in sensitivity over that achieved in the flame, their use did not eliminate all problems involved in analyzing biological samples without pretreatment. These techniques also have inherent background problems. For tube-like furnaces, spectral interferences due to light-scattering and molecular absorption are encountered for samples with complex matrices. The light scattering is caused by condensation of water vapor escaping from the ends of the tube and by formation of inorganic molecules (66). With the carbon rod atomizer similar effects are observed. When flameless devices are used, smoke from residual organic material or salts can enter the sample beam, causing a severe nonspecific background absorption.

But once the above problems have been overcome, flameless atomic absorption is as sensitive a technique as is available for the determination of chromium in biological material.

Flameless atomic absorption was used during the investigation as an aid to evaluation of the results obtained from the two photometric methods developed. A complete discussion of the atomic absorption method is presented in Section VIII of Chapter VI.

CHAPTER III

SAMPLE PREPARATION

The importance of sample preparation in the determination of trace quantities in organic compositions, both natural and synthetic, is widely acknowledged. The subject is vital in biochemical investigations.

The elimination of organic matter with quantitative retention of the sought-for elements in the residue, introduces problems of many facets. These problems have been extensively investigated and documented; but still, when developing a new method of analysis, the technique by which organic matter is eliminated must be considered again with emphasis on the particular situation.

The retention factor during digestion is especially important in the analysis for trace amounts of metals. Minute losses during the digestion step of a macro analysis may have no significant influence on the final result but in trace analysis can cause drastic errors. The study of dry ashing and wet chemical oxidation for the destruction of organic compositions, and the subject of procedural losses of elements to be determined have been documented by J. Pijck et al. (67). The metals involved were As, Sb, Cr, Ca, Au, Fe, Cu, Hg, Pb, Mn, Mo, V, Ag, and Zn. The results of the study by Pijck et al. are summarized in Tables 1 and 2. Dry ashing as a means of digestion in the analysis for chromium, as well as many other metals, must be rejected as a general method because of the considerable loss of metal, even at the relatively low temperature of

Table 1. Metal Recovery after Dry Ashing (67)

Element	% Recovery as a Function of Temperature and Time			
	400°C 24 hours	500°C 12 hours	700°C 6 hours	900°C 3 hours
Antimony	67	81	35	9
Arsenic	23	0	0	0
Chromium	99	99	86	56
Cobalt	98	75	67	30
Copper	100	98	87	58
Iron	86	81	52	27
Gold	19	0	0	0
Lead	103	70	32	13
Manganese	99	96	85	79
Mercury	1	0	0	0
Molybdenum	100	97	85	13
Silver	65	67	45	21
Vanadium	102	99	70	60
Zinc	100	98	69	30

Table 2. Metal Recovery Following Wet Chemical Oxidation (67)

Element	Digested Material - % Recovery				
	Blood	Urine	Powdered Vegetable	Muscular Tissue	Under Total Reflux
Antimony	99	95	95	94	101
Arsenic	93	94	95	93	101
Chromium	100	100	101	100	
Cobalt	100	100	101	99	
Copper	102	101	102	102	
Iron	98	92	95	85	100
Gold	77	100	77	65	101
Lead	100	101	100	100	
Manganese	99	94	98	100	
Mercury	24	87	45	30	100
Molybdenum	101	100	101	100	
Silver	100	100	100	100	
Vanadium	100	100	100	100	
Zinc	99	101	100	99	

700°C. Although this chromium loss could be eliminated by using still lower temperatures, then an extremely long length of time would be required for complete ashing. In addition, all dry ashing procedures for the destruction of organic matter demand usually rather large samples and additional equipment, like electric muffles, platinum dishes, and other apparatus.

A variety of techniques is available for the digestion of organic material. These techniques employ nitric acid, perchloric acid, sulfuric acid, and periodic acid, and various mixtures of these. The acids studied in this investigation were nitric acid, perchloric acid, and sulfuric acid. In their evaluation, main emphasis was placed on 1) speed of digestion, 2) behavior of the digestion system, and 3) the introduction of complications in the final determination. Various mixtures of the three acids were also evaluated on the same three criteria.

The actual digestion was performed in 13 x 100 mm test tubes with calibration marks at 1.0 and 2.0 milliliters. Depending on the physical state of the sample, either 200 μl or 0.200 g of biological material, were placed in calibrated test tubes and the tubes put into the digestion block (the block is described in Section V of Chapter VI). The hot plate used to heat the block was set to the surface temperature necessary to establish the desired temperature in the tubes and the digestion allowed to go to completion. The digestion was judged completed when the solution in the tube was colorless and clear.

Nitric acid was the first acid to be evaluated. Concentrated nitric acid was very fast in destroying the organic matter found in

biological materials. A 200 µl sample of serum was digested with 1 ml of concentrated nitric acid in 90 minutes or less depending on the temperature of the digestion block. But the reaction was not "well behaved." If the temperature of the block was taken above 200°C, the solution in the digestion tubes bumped, sometimes severely enough to cause ejection. When the surface temperature of the hot plate was lowered to prevent vigorous bumping, the time required for complete digestion rose to about five hours. As a further drawback, it was found that nitrate ion severely interferes in the final steps of both methods of determination intended, because of undesirable reactions with the colorimetric reagents.

Sulfuric acid was found to be slower at digesting 200 µl of serum than was nitric acid at the same temperature. But the digestion was considerably more well-behaved, and left open the possibility of operating at a higher temperature. Then, however, a strong tendency prevailed to merely char the organic material in the digestion mixture. The charred material strongly resisted further digestion, requiring as much as ten hours to obtain a colorless solution even with the block temperature at 325°C. But once the digestion was complete, sulfuric acid solutions of digested serum caused no further complications in either method of final determination.

Perchloric acid was the last acid to be studied. The use of perchloric acid for the digestion of organic material demands that all safety requirements be followed. When the safety requirements are followed and common sense is employed, perchloric presents no greater safety hazard than any other acid studied. This acid proved excellent

for the digestion of organic material. In experiments performed on blood serum, perchloric acid completely digested the serum in less than 60 minutes at a block temperature of 225°C. The digestion proceeded in a very well-behaved manner. But the use of perchloric acid, especially at elevated temperatures (> 200°C), caused problems during the final steps of analysis by both colorimetric methods used. These problems will be explained in detail when discussing the two reagents (Chapters IV and V).

The first mixture of acids studied was composed of equal volumes of concentrated nitric acid and concentrated sulfuric acid. This mixture digested the serum in around 60 minutes at a block temperature of 200°C, but the mixture behaved in a manner similar to that of nitric acid, that is, sometimes bumped during the digestion. Bumping could be eliminated by lowering the temperature. But with the block at 125°C, the digestion time increased to three hours. No ill effects in the final determinations are introduced by this acid mixture, obviously because the otherwise interfering nitric acid was completely removed during the digestion.

The next mixture evaluated was one composed of equal volumes of concentrated sulfuric acid and concentrated perchloric acid. The digestion proceeds in a well-behaved manner, but the same problems encountered in the final determination with perchloric acid alone were also caused by this mixture.

The final mixture studied was one composed of the concentrated sulfuric, nitric, and perchloric acids in a volume ratio of 15:3:2.

The digestion of blood serum was complete in less than 60 minutes with the block at 300°C. The reaction produced no bumping and none of the problems encountered otherwise occurred here. Because of the speed

of digestion, well-behaved nature of the reaction, and absence of interferences, the mixture of 75% sulfuric, 15% nitric, and 10% perchloric acids was used as the digesting solution throughout the entire investigation. Any future reference to "the digestion acid" or "the acid mixture" will indicate this mixture.

CHAPTER IV

DIPHENYLCARBAZIDE METHOD

Reagent Properties

Diphenylcarbazide is presently considered the preferred reagent for the colorimetric determination of minute amounts of chromium and has been used in the analysis of rocks, minerals, iron and steel, water, soil, air, leather, and biological materials (68). Under proper conditions, hexavalent chromium reacts with the reagent to produce an intensely red-violet compound of which the formula is not known. The structure of diphenylcarbazide is given in Figure 1 and the absorbance curve of the chromium(VI)-diphenylcarbazide compound in Figure 2. The molar absorptivity of the colored compound, calculated on the basis of the molarity of dichromate, is known to be 31,400 l/mole-cm at the absorption maximum of 540 nm (68). Many metals such as mercury, copper, cadmium, silver, lead, nickel, cobalt, manganese, zinc, and iron also react with the reagent, giving colors ranging from red through violet to blue. But under the conditions of low pH, as required for the determination of chromium, the sensitivity of the color reaction for the above metals is so small that the method may be regarded as practically specific for chromium.

Long-Path Microcells

In biological material the chromium is present in such small amounts and concentrations that despite the high absorptivity involved

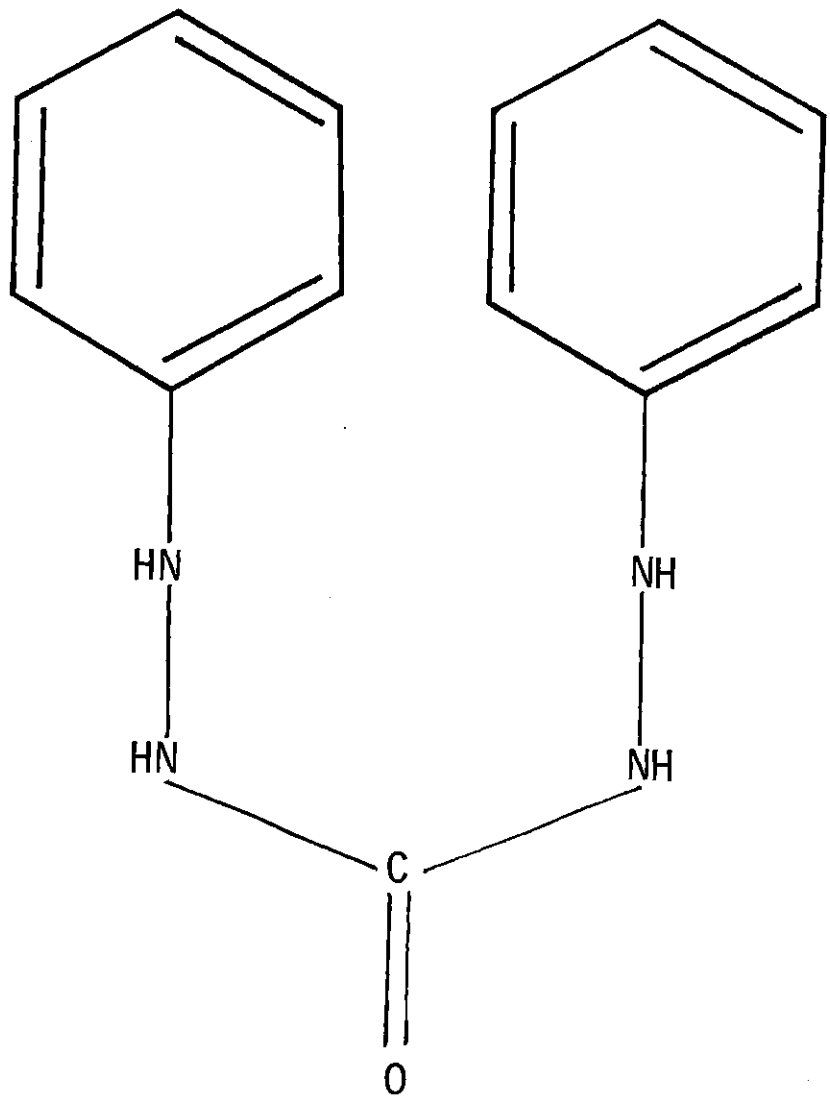

Figure 1. Structure of Diphenylcarbazide

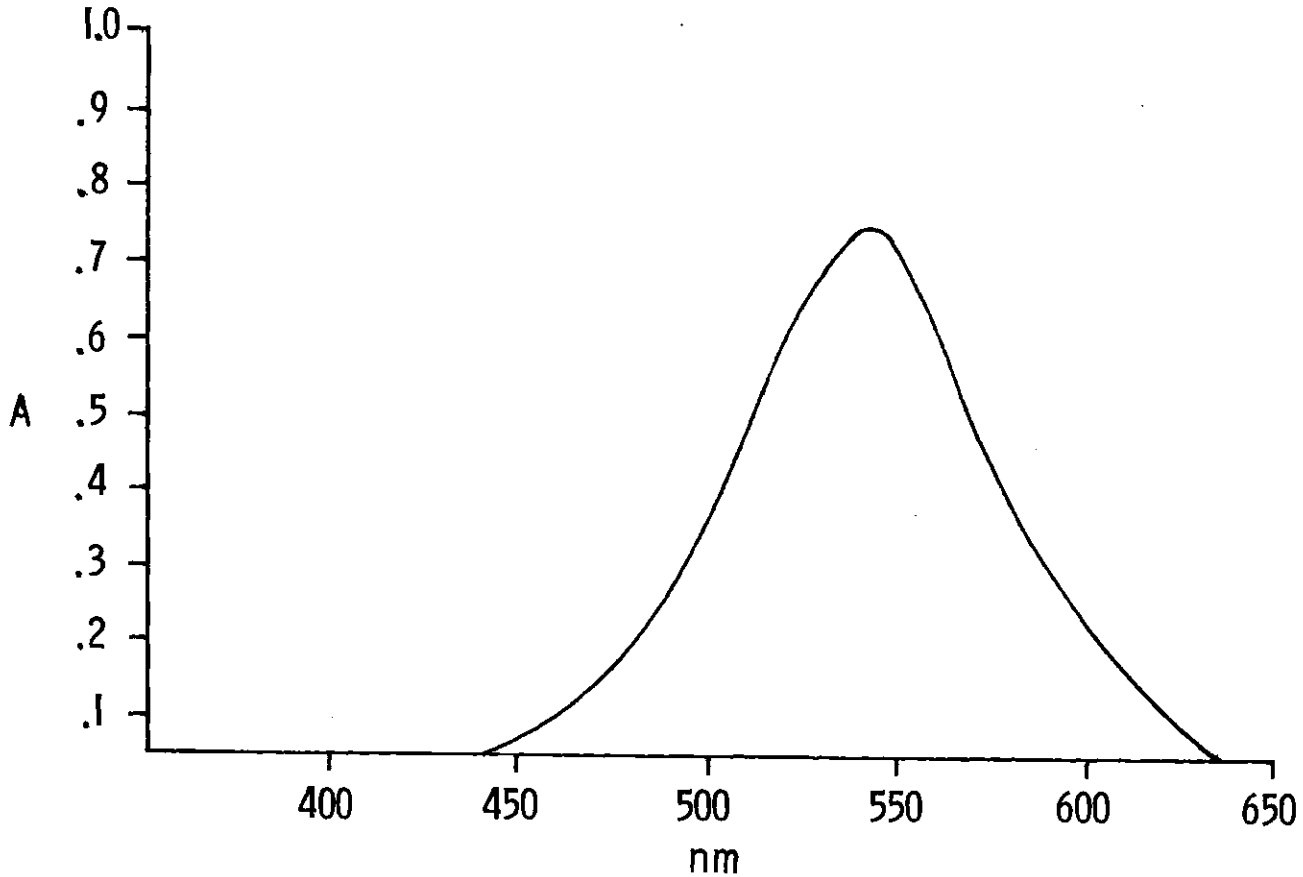

Figure 2. Absorption Curve of Chromium(VI)-Diphenylcarbazide

such large quantities of sample material are required that it is difficult to use the existing methods in routine diagnostical analysis. This is at least the situation when the usual photometric equipment is employed. However, with the use of long-path microcells the sample size is no longer so restricting a problem.

In photometry commonly 1-cm cells or equivalent tubes are used. Some higher priced instruments will accept cells longer than one centimeter but the increase in path length is paid for with a disproportionate increase in the volume of solution required to fill the cell. When the amount of sample is not limited no problem exists, but when the sample volume is restricted the dilution necessary to reach the volume requirements can cancel any increase in the absorbance resulting from the longer path. Therefore, if the use of long-path cells is to be of advantage, the lengthening of the path must be achieved without an offsetting volume increase. The theoretical and practical considerations related to this problem are found in the synopses on the philosophy of long-path and microcells in the theses of Barnes (69) and Paschal (70) as well as in several papers by Flaschka and co-workers (71,72).

It is very instructive to demonstrate the improvements in the determination of chromium in blood serum derived from the use of long-path microcells. Assume the following case: A 1-ml sample of serum known to contain 25 ng/ml (4.8×10^{-7}M) is processed in such a way that the volume of the final sample preparation is also one milliliter.

Then, the absorbance due to the chromium(VI)-diphenylcarbazide is 0.015, when using a 1-cm cell. Measurement of such a low absorbance is "pushing the point" with most photometers. Therefore, something must be

done in order to obtain a larger absorbance reading. There are three avenues open to resolve this problem. First, more sample is used, second, the volume of the final sample preparation is lowered, and third, a long-path microcell is employed. The first avenue, that is, increasing the sample size, is applied in presently existing procedures that employ diphenylcarbazide (73). These methods, depending on the expected chromium concentration use five to ten milliliters of blood or serum. Large sample sizes can be tolerated for research purposes but for clinical testing, one milliliter is already often to be considered quite high. The second approach, reduction of the volume of the final sample preparation, is not practically feasible. When using conventional 1-cm cells for the absorbance measurement, one milliliter is barely enough for adequate filling. There is an additional difficulty, namely to digest a 10ml serum sample, add the required reagents, and still obtain a volume of final sample preparation of less than one milliliter. The third avenue, use of long-path microcells, solves all of the problems described. Assuming the same case as above, the absorbance due to the chromium-diphenylcarbazide is 0.30 when measured in a 20-cm microcell. With this large absorbance, there also exists the possibility of even decreasing the sample volume well below one milliliter. A series of experiments and calculations were performed to determine the minimum volume of sample and final sample preparation that would befit the following variety of requirements: conditions needed for digestion, kinetics of all reactions involved, restrictions to be placed on sample volume, and a certain minimum absorbance reading. The conclusion was that a sample volume of 0.2 ml and a 2-ml final sample preparation are well suited, and for a serum containing

25 ng/ml chromium, give an absorbance reading of a comfortable 0.03.

Method

With the question of volume size thus settled, the development of the actual procedure began.

The first step in any colorimetric method for the determination of chromium in biological material is the destruction of the organic matrix. The problems connected with this task have already been discussed in Chapter III dealing with sample preparation. There, it was also shown that the best choice for a diphenylcarbazide finish proved to be digestion by a mixture of concentrated sulfuric, nitric, and perchloric acids in a volume ratio of 15:3:2.

As mentioned before, chromium(III) is the primary form of chromium found in biological material, but diphenylcarbazide reacts only with chromium(VI), consequently the chromium must be oxidized to the hexavalent state. There are many reagents available for this oxidation, among them, perchloric acid, and persulfate, bismuthate, and permanganate ions. Experiments were performed with each of these to determine which was the best to be used.

At a first glance, perchloric acid would seem to be the most advantageous reagent. Besides being the oxidizing agent, the perchloric acid can also be used as the digestion acid. Hot, concentrated perchloric acid has an oxidation potential of approximately +2 volt but the oxidizing power of the hot, concentrated acid is voided by cooling or diluting.

The situation appears to be ideal; the hot perchloric acid digests the biological material, then oxidizes the chromium(III) to chromium(VI)

and finally looses its oxidizing power simply by cooling. In actual practice, however, problems occur that complicate matters. The hot perchloric acid does oxidize the chromium(III) to chromium(VI) but at the same time, small amounts decompose according to the following reaction:

$$2HClO_4 \xrightarrow{Heat} Cl_2 + 3O_2 + H_2O_2$$

The hydrogen peroxide thus produced reduces some chromium(VI) to chromium(III). This reduction leads to low chromium recovery. It is known, e.g. from steel analyses, that the amount of hydrogen peroxide formed during the decomposition reaction can be minimized by rapidly cooling the solution from the working temperature to near room temperature. Experiments were performed to establish the extent to which the reduction takes place both in chilled and unchilled solutions. Chilling the solutions increases the chromium recovery, but still never to better than 95 percent. But of even more importance was the inconsistency of the loss. The recovery ranged from a low of 60 percent in unchilled solutions to a high of 95 percent in chilled solutions. The details and results of the experiments are given in Section 1 of Chapter VI.

Persulfate readily oxidizes chromium(III) to chromium(VI). The excess of persulfate is easily eliminated by boiling the solution. Unfortunately, here, too small amounts of hydrogen peroxide are generated, again causing a low recovery of chromium.

It has been reported (73) that the same problem encountered with perchloric acid and persulfate ion is also experienced with bismuthate.

In addition, the excess bismuthate must be removed by filtration.

In acid solution, permanganate oxidizes chromium(III) to chromium(VI) according to the following reaction:

$$10Cr^{3+} + 6MnO_4^- + 11H_2O \rightarrow 5Cr_2O_7^{-2} + 6Mn^{2+} + 22H^+$$

Saltzman was the first to propose permanganate ion oxidation in the determination of chromium with diphenylcarbazide (73).

An analogous set of experiments as was employed with the perchloric acid oxidation was used to study the recovery of chromium with permanganate. The results showed that with permanganate as the oxidizing agent the recovery of chromium is always higher than 99 percent. Details of the experiments are listed in Section II of Chapter VI.

Once the chromium has been oxidized to the hexavalent state the excess permanganate must be eliminated before the diphenylcarbazide is added. Sodium azide can be used to reduce any manganese(VII) to manganese(II). The excess azide is destroyed by heating.

The diphenylcarbazide is dissolved in acetone and diluted with an equal volume of 0.1 F sodium dihydrogen phosphate. The phosphate is added because, according to Saltzman (73), it stabilizes the color of the chromium-diphenylcarbazide.

Following the color development, the final step is the measurement of the absorbance, that has to be done against a reagent blank.

There are two possibilities for determining the chromium concentration in the final sample preparation. One is a calibration curve which from the point of view of routine analysis, is the preferred way. Besides

being the simplest method, it is sometimes the only method when the amount of sample is limited. But, matrix effects sometimes necessitate the use of one other method which is less affected by sample composition. This second method is standard additions which eliminates many of the problems associated with complex samples.

Experiments were performed to decide which procedure would be required for determining the concentration of chromium by the diphenylcarbazide method. It was found that the results obtained were not affected by the procedure by which they were obtained. Thus, the simple approach via a calibration curve can be employed in routine diagnostic testing. Complete details and results of the above experiments are discussed in Section IV of Chapter VI.

Using the facts and data presented above, a procedure has been developed for the determination of chromium in biological material. It is presented in working procedural form in Section III of Chapter VI.

Evaluation of Results

Precision

To test for the precision, the following scheme was adopted. Three serum pools were at hand and triplicate samples were taken from each pool and processed simultaneously with three different chromium standards and one reagent blank. The latter was used to set 100 percent transmittance. The data from the standards were used to establish a calibration curve and from it were obtained individual values for each triplicate. These values were averaged with any obvious run-away results discarded. Several sets of triplicate analysis were run on each pool.

The results are presented in Tables 3, 4, and 5.

As can be seen the precision is quite satisfactory. The spread within one triplicate is low, and run-aways are quite rare. Thus in actual practice with time, working room, equipment, and sample material at a premium, taking only a single sample and reading, will give results of high reliability.

Figure 8 shows the data for a series of standards. Their precision of course is implicit in the overall values discussed above. But an additional separate evaluation is in order and important. Because of the high precision, it is readily permitted in practice to establish a calibration curve once and then to use it until preparing a new set of reagent solutions or other conditions make recalibration necessary. Of course, adherence to the practice of regularly checking the validity of the calibration curve by incorporating a standard every so often is advisable.

Accuracy

The reliability of the diphenylcarbazide method has been proven in many works including those related to biological material. In the scaled-down version proposed here, the accuracy has been established in preliminary experiments where known amounts of dichromate were given and correctly found.

The oxidation efficiency was tested by taking known amounts of dichromate, reducing, and analyzing involving reoxidation. Also here, the amount given was correctly found.

Because the biological material was digested for destruction of all organic matter, only the remaining inorganic matter had to be considered for possible interference. From what is known of the diphenylcarbazide

Table 3. Determination of Chromium in Serum Pool I by the Diphenylcarbazide Method

Experiment #	Triplicate Results (ng/ml)	Average (ng/ml)
1	25.0, 25.3, 25.3	25.2
2	26.2, 26.1, 25.4	25.9
3	27.4, 27.1, 27.6	27.4
4	26.6, 26.7, 26.6	26.6
5	28.3, 28.3, 28.6	28.4
6	27.0, 27.3, 27.6	27.3
7	24.5, 24.8, 25.4	24.9
8	25.0, 25.6, 25.6	25.4
9	26.8, 26.4, 26.5	26.6
10	25.1, 24.5, 49.8	24.8
11	26.3, 26.5, 26.4	26.3

Average Value 26.3 ng/ml

Standard Deviation ± 1.2 ng/ml

Table 4. Determination of Chromium in Serum Pool II by the Diphenylcarbazide Method

Experiment #	Triplicate Results (ng/ml)	Average (ng/ml)
1	27.7, 27.1, 27.2	27.3
2	28.8, 29.0, 29.8	29.2
3	25.3, 25.4, 25.3	25.3
4	25.3, 25.9, 26.2	25.8
5	26.4, 26.0, 26.6	26.3
6	27.0. 27.1, 27.1	27.1
7	28.0, 28.5, 28.6	28.4
8	27.0, 27.1, 27.4	27.3
9	29.1, 29.3, 29.6	29.3
10	26.5, 26.6, 26.8	26.7

Average Value 27.3 ng/ml

Standard Deviation ± 1.4 ng/ml

Table 5. Determination of Chromium in Serum Pool III by the Diphenylcarbazide Method

Experiment #	Triplicate Results (ng/ml)	Average (ng/ml)
1	22.6, 22.4, 22.2	22.4
2	22.4, 22.3, 22.3	22.3
3	21.9, 21.9, 21.6	21.8
4	23.7, 24.3, 24.2	24.1
5	24.6, 24.7, 24.7	24.7
6	24.9, 24.1, 24.3	24.4
7	22.9, 21.9, 83.6	22.4
8	23.4, 23.7, 23.8	23.6
9	22.2, 22.2, 22.3	22.2
10	23.0, 23.1, 23.2	23.1
11	21.9, 21.6, 21.7	21.7
12	22.4, 23.1, 23.2	22.9
13	23.4, 23.9, 23.8	23.7
14	24.3, 23.6, 62.7	24.0
15	22.4, 22.9, 22.8	22.7
16	21.6, 21.0, 20.9	21.2

Average Value 23.0 ng/ml

Standard Deviation ± 1.0 ng/ml

method from other analytical applications none of the inorganic serum ions should have any effect. To verify this expectation standard additions of chromium were made in two ways and the results thus obtained compared with those stemming from the new procedure. A series of experiments were performed in which two aliquots of a serum were processed one with and one without addition of a known amount of chromium. In all cases the amount added was recovered satisfactorily. In another series, several aliquots of serum were taken and processed as follows, one with no chromium added and the others with increasing amounts of chromium. Then the usual plotting and extrapolation to the negative side of the x-axis was performed as shown in Figure 3. Again the chromium value obtained was a perfect match with that from the proposed procedure.

To completely eliminate any doubt regarding a newly developed method, of course, requires comparison with results obtained by another method, which should operate on a completely different principle. Of such methods for the present work, only flameless atomic absorption was available and was used for the ultimate test.

Initially, it was hoped that the flameless atomic absorption analysis could be performed on untreated serum. But unfortunately, experiments proved this not to be feasible and consequently the serum was digested, according to the details outlined in Chapter III. Before digestion, certain of the samples were spiked with known amounts of chromium. The samples were then processed as outlined in Section VIII of Chapter VI. The chromium was thus determined by the method of standard additions.

A comparison of the results obtained by flameless atomic absorption and those using diphenylcarbazide is presented in Table 6. As can be seen

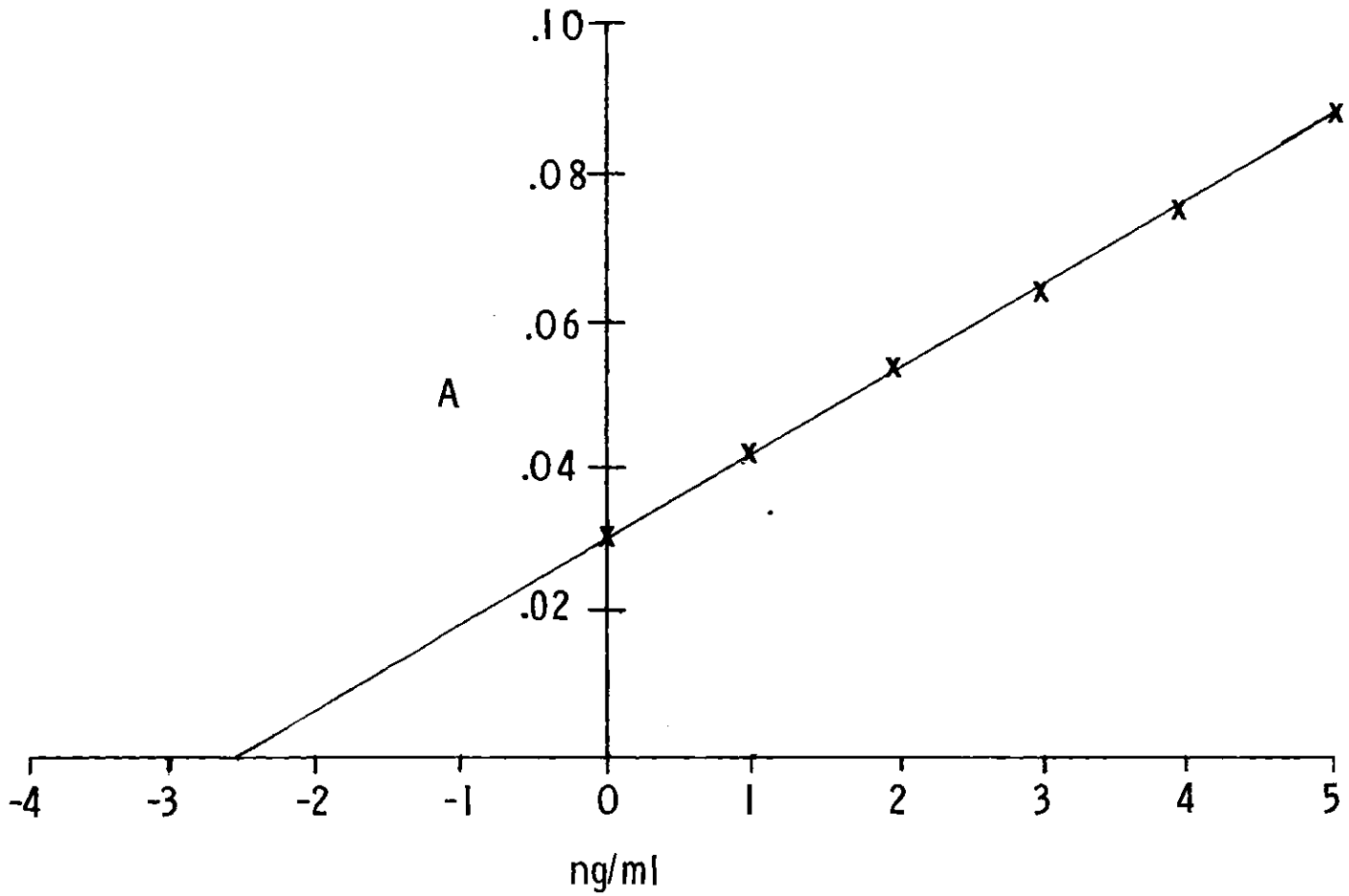

Figure 3. Chromium by the Method of Standard Addition

Table 6. Comparison of the Results Obtained by the Diphenylcarbazide and Flameless Atomic Absorption Methods

	Serum I	Serum II	Serum III
Diphenylcarbazide Method			
Average Value (ng/ml)	26.3	27.3	23.0
Standard Deviation (ng/ml)	± 1.2	± 1.4	± 1.0
Atomic Absorption Method			
Average Value (ng/ml)	25.6	27.0	22.1
Standard Deviation (ng/ml)	± 1.1	± 1.2	± 1.4

the results compare very favorably. Although the atomic absorption values seem to be slightly lower than the diphenylcarbazide values, calculations show that on the 95 percent confidence limit there is no statistical difference between the averages.

Interference Study

The above results were obtained from pooled serum which can be assumed to have normal concentration of all ions. The question still remains as to the effect of abnormally high concentrations of these ions on the results. Therefore a study was performed to determine this effect. As all anions are destroyed or removed, emphasis was only needed on the cations. The answer as to the situations at abnormally elevated concentrations was answered by experiments. Of the elements in question, iron, copper, zinc, calcium, and magnesium were investigated and no interference encountered, that is, there was no change in the values obtained or the spread within triplicate analyses. Tests were made only up to 20 times the normal level.

CHAPTER V

o-TOLIDINE METHOD

The chromium(VI)-diphenylcarbazide complex with a molar absorptivity of 31,400 l/mole-cm appears to be extremely well suited for the photometric determination of minute amounts of chromium. But closer examination reveals some drawbacks. The above absorptivity is based on the molarity of dichromate. When the calculation is made on the basis of the molar concentration of chromium (instead of dichromate) the "apparent absorptivity" of the chromium-diphenylcarbazide complex becomes 15,700 l/mole-cm (because of 2 Cr per $Cr_2O_7^{-2}$). This is not a value that allows a reagent to be considered superior. Therefore, a search was instigated for other reagents. Two possible compounds are 1-(2-pyridylazo)-2-naphthol (PAN) and 4-(2-pyridylazo)-resorcinol (PAR). Both form complexes with chromium(III) that have (true) molar absorptivities around 45,000 l/mole-cm. Unfortunately the reagents lack selectivity as they form strongly absorbing complexes with all of the heavy metals found in biological material. Thus, a preliminary separation of the chromium would be necessary, a fact that makes PAN and PAR less desirable for routine analysis.

Chemical Amplification

Several other reagents were considered but rejected because of inadequacies regarding sensitivity or selectivity or because of other difficulties to be expected in application in routine analysis. There

exists, however, one particular approach to increase sensitivity that has found growing attention in recent years, namely, chemical amplification. The principle applied to the present situation is: instead of operating on a one-by-one basis as is the case between dichromate and diphenylcarbazide or chromium and PAN or PAR, use is made of the change of three electrons per chromium when going from chromium(VI) to chromium(III). If these electrons could be used to cause formation of a strongly absorbing species the apparent absorptivity per mole of chromium would be triple that corresponding to the one-to-one basis. One of the possibilities in this respect is the reaction of dichromate with iodide leading to the liberation of three moles of iodine per mole of chromium followed by extraction of the liberated iodine into chloroform or carbon tetrachloride to give the well known deeply violet solution. This is only an example for amplification because the absorptivity of iodine is too low to yield an apparent absorptivity that would be sufficient to determine chromium at levels normally occurring in serum. However, the search in the direction of amplification lead to a paper by Barek and Berka (75) that seemed to offer great possibilities. These authors developed a new method for standardizing ascorbic acid solution employing potassium dichromate as the primary standard. The method works as follows: a known amount of potassium dichromate is dissolved and to the solution is added manganese(II) pyrophosphate, which is oxidized to the manganese(III) pyrophosphate. Next is added o-tolidine which is reversibly oxidized by the manganese(III) pyrophosphate to the corresponding intensely yellow colored quinonediimine, 4,4'-diimino-3,3'-dimethyl-biphenoquinone. This is then titrated with ascorbic acid to a visual end point. The reactions involved are presented in Figure 4.

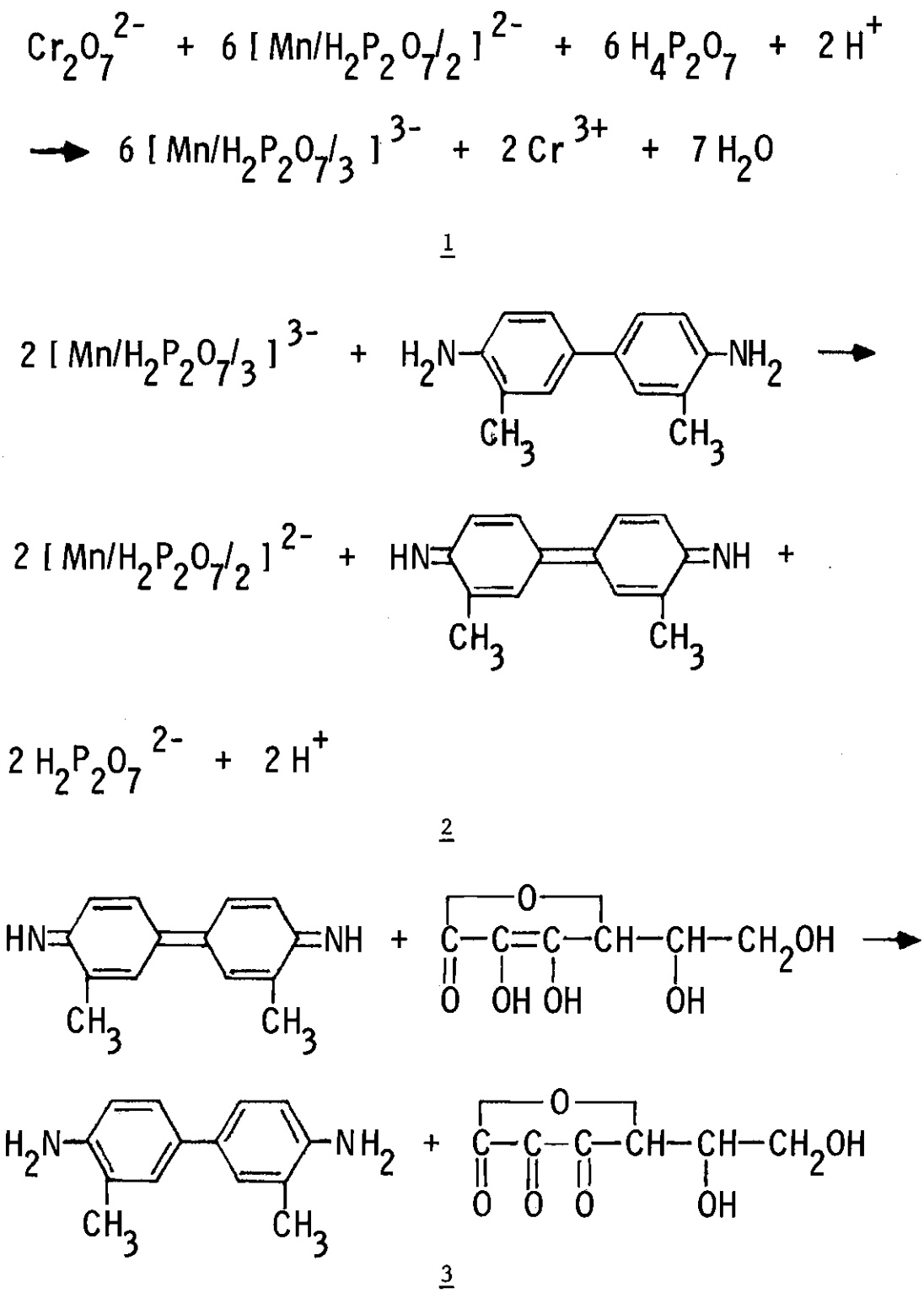

Figure 4. Chromium(VI) Reaction Sequence

Properties of the Quinonediimine of o-Tolidine

Barek and Berka did not develop the method with the intent of amplification, but rather to place the reaction between dichromate and ascorbic acid on a predictable and reproducible stoichiometric basis which is not realized when letting the two components react directly. However, the procedure contains a promising possibility of amplification, with respect to the determination of chromium(III) in biological material. After oxidation to chromium(VI) the first two reactions of the scheme in Figure 4 are allowed to take place and then the concentration of the quinonediimine is established by photometry because for a titration, the amounts involved are far too small. From the scheme in Figure 4, it can be deduced that 1.5 moles of diimine are formed per one mole of chromium. Preliminary experiments (for details see Section VI of Chapter IV) established the molar absorptivity of the quinonediimine at the absorbance maximum of 440 nm as 30,200 l/mole-cm, which is quite close to the 31,400 l/mole-cm of the chromium(VI)-diphenylcarbazide. But the apparent absorptivity of 30,200 l/mole-cm $\times$ 1.5 = 45,300 l/mole-cm per mole of chromium is about three times that of the diphenylcarbazide compound (15,700 l/mole-cm).

Method

With these promising facts at hand the development of a procedure was started. As already pointed out the chromium has to be in the hexavalent state in order to start the sequence of reactions presented in Figure 4. The hexavalent state is also the basis for the diphenylcarbazide

method, and, therefore, the chances were good that the digestion and oxidation steps already established for the proceeding method would be adequate for the new one. Experiments fully verified this expectation. The oxidation of the chromium(III) with permanganate and the subsequent reduction of excess permanganate provides also the required manganese(II), so only pyrophosphate need be added. The final steps are then addition of o-tolidine and measurement of the absorbance.

Using the above information, a working procedure for determination of chromium in biological material was developed. This procedure is presented in Section VII of Chapter VI.

Evaluation of Results

The three serum pools employed in the diphenylcarbazide and flameless atomic absorption studies were analyzed for chromium using the procedure outlined above. As in the diphenylcarbazide study, individual samples from each pool were processed in triplicate and the three results averaged. Obvious run-away values were not included in this average. Three known chromium solutions were processed along with the three serum samples, as was a reagent blank. The chromium standard solution was used to establish a calibration curve and the reagent blank used to set 100 percent transmittance on the photometer.

The results of repeated triplicate analyses were averaged and an estimate of the standard deviation calculated. These results for all serum pools are shown in Tables 7, 8, and 9.

The average chromium values for each pool as determined by the o-tolidine, diphenylcarbazide, and atomic absorption method are listed in Table 10. Comparison of these results reveals a slight but seemingly

Table 7. Determination of Chromium in Serum Pool I by the o-Tolidine Method

Experiment #	Triplicate Results (ng/ml)	Average (ng/ml)
1	29.0, 29.1, 29.4	29.2
2	29.0, 28.6, 28.5	28.7
3	29.2, 29.3, 29.5	29.4
4	27.3, 27.6, 27.9	27.6
5	31.8, 31.8, 31.9	31.8
6	30.1, 30.0, 30.5	30.2
7	29.5, 29.5, 29.8	29.6
8	30.9, 30.6, 30.9	30.8
9	28.1, 28.6, 27.9	28.1

Average Value 29.5 ng/ml

Standard Deviation ± 1.3 ng/ml

Table 8. Determination of Chromium in Serum Pool II by the o-Tolidine Method

Experiment #	Triplicate Results (ng/ml)	Average (ng/ml)
1	31.0, 31.0, 31.6	31.2
2	30.6, 30.6, 30.8	30.7
3	33.2, 34.0, 92.8	33.6
4	30.6, 30.4, 30.0	30.3
5	32.6, 32.7, 32.8	32.7
6	30.3, 30.1, 30.5	30.3
7	31.3, 31.7, 31.1	31.4
8	32.0, 32.2, 32.4	32.2

Average Value 31.6 ng/ml

Standard Deviation ± 1.2 ng/ml

Table 9. Determination of Chromium in Serum Pool III by the o-Tolidine Method

Experiment #	Triplicate Results (ng/ml)	Average (ng/ml)
1	24.1, 24.9, 24.9	24.7
2	25.7, 25.5, 25.9	25.7
3	24.7, 24.7, 25.1	24.9
5	26.0, 26.3, 26.4	26.2
5	28.4, 28.4, 28.4	28.4
6	29.0, 29.2, 29.1	29.1
7	24.3, 25.0, 24.8	24.7
8	25.3, 25.0, 25.6	25.3
9	26.3, 26.4, 26.5	26.4

Average Value 26.2 ng/ml

Standard Deviation ± 1.6 ng/ml

Table 10. Average Chromium Values from o-Tolidine, Diphenylcarbazide, and Atomic Absorption Methods

		Serum I	Serum II	Serum III
o-Tolidine Method				
Average Value	(ng/ml)	29.5	31.6	26.2
Standard Deviation	(ng/ml)	± 1.3	± 1.2	± 1.6
Diphenylcarbazide Method				
Average Value	(ng/ml)	26.3	27.3	23.0
Standard Deviation	(ng/ml)	± 1.2	± 1.4	± 1.0
Atomic Absorption Method				
Average Value	(ng/ml)	25.6	27.0	22.1
Standard Deviation	(ng/ml)	± 1.1	± 1.2	± 1.4

definite trend toward higher values for the chromium concentrations when the o-tolidine method is used, although on a 99% confidence level there is no statistical difference between the averages.

As in the diphenylcarbazide method, experiments were performed that tested the effect of abnormally high concentrations of iron, copper, zinc, calcium, and manganese on chromium recovery in the o-tolidine method. These metals did not affect the results even when the concentrations were 20 times greater than those normally found in man.

Possible Interference by Vanadium

There still exists the problem of explaining the slightly yet consistently higher results obtained with the o-tolidine method. Since the more commonly found metals in serum did not have any effect, the possibility had to be considered that another trace metal may be responsible. Since the method is based on redox reactions a survey of electrochemical potentials revealed vanadium as a possible source for the difference. A simple experiment verified the possibility. When o-tolidine was added to a solution containing vanadium(V) and manganese(II) pyrophosphate the solution turns yellow immediately. Serum vanadium if not already present as vanadium(V) is oxidized to the pentavalent form during the permanganate oxidation step. This vanadium(V) then oxidizes manganese(II) pyrophosphate to manganese(III) pyrophosphate with the formation of vanadium(IV). The manganese(III) thus produced initiates the rest of the reaction sequence shown in Figure 4. The final result is an apparent increase in the chromium concentration. Only one manganese(III) is produced per vanadium(V) and consequently only 0.5 moles of the quinonediimine

occur per mole vanadium. On a weight basis approximately nine nanograms of vanadium per milliliter serum is required to cause the difference observed between the results from the diphenylcarbazide and o-tolidine methods. When serum samples were actually spiked with nine nanograms of vanadium per milliliter the results for the chromium did come out higher by 2.9 nanograms per milliliter.

Due to lack of appropriate facilities it was not possible to actually determine the vanadium in the sera at hand. However, because the sera were three pooled ones the assumption that the vanadium levels were normal was a reasonable one. With the normal level for vanadium in human serum reported to be 4-9 nanograms per milliliter (77) the slightly elevated chromium values obtained by the o-tolidine method find an acceptable explanation.

The o-tolidine method presented here is approximately three times as sensitive as the diphenylcarbazide method; the reagent solutions are easier to prepare, and show better stability; so does the color developed. Thus the new method offers distinct advantages. But due to the still somewhat unclear situation concerning vanadium (or another culprit) a word of caution is in order, when planning application of the method to routine diagnostic testing.

A low chromium value obtained by this method definitely indicates a low chromium in the patient regardless of the vanadium concentration. But an extremely high vanadium concentration could bring a de facto low chromium value up to the normal-level range. An apparent high chromium value could be due to an extreme vanadium concentration. But, fortunately,

the high values are of less interest because high chromium states usually are due to poisoning, which normally manifests itself in clear clinical symptoms.

Thus the situation is not totally unfavorable but until more is known about vanadium and especially its extreme highs, the role this metal can play should be borne in mind.

CHAPTER VI

EXPERIMENTS

Many facts established experimentally have been mentioned in the preceding sections. There, only the results were of interest and the detailed description of the actual experiments would have interrupted the smooth flow of thoughts. For the evaluation of the results, however, the procedural details are of importance and are given here.

Section I

"Chromium Recovery with Perchloric Acid Oxidation"

The difficulty of fully oxidizing chromium with perchloric acid is well known, but in several branches of analytical chemistry, e.g. steel analysis, procedures have been developed that eliminate the problem. They prescribe a rapid chilling by inserting the heated vessel into an ice bath. Then, the temperature range within which the formation of the detrimental hydrogen peroxide predominates is quickly transgressed and correct results are obtained.

Application of this principle was tested as described below but to no avail. A volume of 5, 10, 25 μl of 6.7×10^{-5}M chromium(III) was placed into three digestion tubes. One milliliter of concentrated perchloric acid was added to each tube. Next the tubes were heated at 200°C for one hour and then allowed to cool to room temperature. The solutions were

diluted with 0.5 ml of deionized water and 0.50 ml of a 0.25% diphenylcarbazide solution was added. The solutions were diluted to two ml with deionized water and the absorbance measured at 540nm. The amount of chromium was obtained from a calibration curve. The curve was prepared from data obtained by taking 5, 10, and 25 μl of a potassium dichromate solution 8.6×10^{-6}M with respect to chromium, adding 0.50 ml of 0.25% diphenylcarbazide, diluting to two ml with deionized water, and measuring the abosrbance at 540nm. Three more solutions exactly like those above were prepared. Once the oxidation was complete, ice made from deionized water was added. This rapidly cooled the solution from 200°C to approximately 50°C. The cooling from 50°C to room temperature was gradual. Diphenylcarbazide was added and the absorbance measured, and the amount of chromium obtained from the calibration curve.

The results are presented in Table 11. They show that without chilling, at the most only 90% of the chromium(III) added is found. To make matters worse the percent recovery was not constant, ranging from 60 to 90%. With chilling, the spread was reduced and the recovery improved, but still did not exceed 95%.

Some minor variations in the approach did not bring any improvement. While it may be possible to resolve the problems, it was felt that the possible need of special equipment for and extra care during the oxidation with perchloric acid would impose difficulties on a practical procedure. Since there are other methods for the oxidation, this particular investigation was terminated.

Table 11. Chromium Recovery Following Perchloric Acid Oxidation

Run #	µl	ng Cr taken	ng Cr found	% Cr recovered
		Without Chilling		
I	5	2.3	1.4	61
	10	4.6	3.8	83
	25	11.5	10.3	90
II	5	2.3	1.7	74
	10	4.6	3.3	72
	25	11.5	10.0	87
III	5	2.3	2.0	87
	10	4.6	3.5	76
	25	11.5	9.7	84
		With Chilling		
I	5	2.3	2.1	91
	10	4.6	4.0	87
	25	11.5	10.9	95
II	5	2.3	2.0	87
	10	4.6	4.0	91
	25	11.5	10.7	93
III	5	2.3	1.7	74
	10	4.6	3.9	85
	25	11.5	10.6	92

Section II

"Chromium Recovery with Permanganate Oxidation"

Another well established method for the oxidation of chromium(III) employs permanganate. While mere cooling suffices in the case of perchloric acid to "remove excess of oxidant," for the permanganate a further reagent is needed for this purpose. Several are at hand, with formic acid and sodium azide being the ones most frequently mentioned in the literature. For the experiments here described sodium azide was selected. The approach was analogous to that used in the perchloric acid study. Solutions were prepared that contained 5, 10, and 25 μl of 8.6×10^{-5}M chromium. A volume of 0.5 ml of 0.1F sulfuric acid was added to each, followed by 0.2 ml of 0.05 F potassium permanganate. The solutions were heated in a boiling water bath for ten minutes, allowed to cool, 0.1F sodium azide added dropwise until the permanganate color disappeared, and heated in the bath five minutes more. After the solutions had cooled, 0.5 ml of 0.25% diphenylcarbazide was added, the solution diluted to two ml, and the absorbance at 540nm measured. The amount of chromium was calculated from the calibration curve described in Section I.

The results are given in Table 12 and show that with permanganate as the oxidant more than 99% of the chromium is recovered.

Section III

"Working Procedure for the Determination of Chromium in Biological Materials Using Diphenylcarbazide"

Table 12. Chromium Recovery Following Permanganate Oxidation

Run #	µl	ng Cr taken	ng Cr found	% Cr recovered
I	5	2.3	2.3	100
	10	4.6	4.6	100
	25	11.5	11.5	100
II	5	2.3	2.3	100
	10	4.6	4.6	100
	25	11.5	11.5	99
III	5	2.3	2.3	100
	10	4.6	4.6	100
	25	11.5	11.5	100

Apparatus and Reagents

1) Calibrated Digestion Tubes. Use 13 X 100 mm pyrex test tubes and calibrate as follows. Place the dry tubes in an exactly upright position and dispense into them 1.00 ml of water, mark the meniscus with a diamond stylus. Add another 1.00 ml of water and again mark.

2) Digestion Block. See Section V of this chapter.

3) Photometer. See Section V of this chapter.

4) Digestion Acid. Mix 75 ml of concentrated sulfuric acid with 15 ml of concentrated nitric acid and 10 ml of concentrated perchloric acid.

5) Water. All water used during the procedure must be deionized.

6) Potassium Permanganate Solution, 0.05 F. Dissolve 0.79 g of reagent grade potassium permanganate in 50 ml of water and dilute to 100 ml. This solution must be prepared weekly.

7) Sodium Azide Solution, 0.1 F. Dissolve 0.65 g of reagent grade sodium azide in 50 ml of water and dilute to 100 ml. This solution must be prepared every three days.

8) Chromium Stock Solution, 20 ng/μl. Dissolve 56.58 mg of reagent grade potassium dichromate (dried at 110°C for one hour) in 100 ml of water and dilute to one liter. This solution will keep indefinitely.

9) Chromium Working Solution, 0.2 ng/μl. Dilute 1.00 ml of the chromium stock solution to 100 ml. This solution must be prepared the day it is to be used.

10) Sodium Dihydrogen Phosphate Solution, 0.1 F. Dissolve 12 g of reagent grade sodium dihydrogen phosphate dekahydrate in 100 ml of water and dilute to one liter. This solution will keep indefinitely.

11) Diphenylcarbazide Solution, 0.25%. Dissolve 0.25 g of certified s-diphenylcarbazide in 50 ml of spectrograde acetone and dilute with 50 ml of 0.1 F sodium dihydrogen phosphate. The diphenylcarbazide solution must be prepared the day it is to be used.

Procedure

1) Place 0.200 ml of a liquid or 200 mg of a solid sample into a calibrated digestion tube.

2) Add one milliliter of digestion acid, place into the heated block (300°C), and digest for one hour.

3) Remove the tube from the block, allow to cool, dilute with 0.5 ml of H_2O, add 0.1 ml of 0.05 F $KMnO_4$, and place in a boiling water bath for 10 minutes.

4) Cool to room temperature and dropwise add 0.1 F NaN_3 until the permanganate color is discharged; add one more drop and heat the tube in boiling water for five minutes.

5) Cool to room temperature, add 0.5 ml of 0.25% diphenylcarbazide solution, and dilute to 2.0 ml with H_2O.

6) Wash the cell with two 0.3 ml portions of solution, fill the cell and measure the absorbance at 540 nm, then fill the cell a second time and measure the absorbance again and average the results. All absorbances are measured against a reagent blank.

7) Obtain the chromium concentration of the sample from a calibration curve which is prepared by carrying 5, 10, 15, 20, and 25 μl of chromium working solution through steps 1 through 6 and plotting the data obtained.

Section IV

"Working Procedure for the Determination of Chromium in Biological Materials Using o-Tolidine"

Apparatus and Reagents

1) - -
2)
3)
4) These steps are the same as respectively listed in the
5) Diphenylcarbazide Method (Section III of this chapter).
6)
7)
8)
9) - -

10) Sodium Pyrophosphate Solution, 0.5 F. Dissolve 22.5 g of reagent grade sodium pyrophosphate dekahydrate in 50 ml of water and dilute to 100 ml. This solution is stable indefinitely.

11) o-Tolidine Solution, 0.005M. Dissolve 0.1 g of certified o-tolidine in 50 ml of water, add one ml of concentrated sulfuric acid and dilute to 100 ml. This solution should be prepared weekly.

Procedure

1) - -
2) These steps are the same as respectively listed in the
3) Diphenylcarbazide Method (Section III of this chapter).
4) - -

5) Cool to room temperature and add 0.1 ml of 0.5 F $Na_4P_2O_7$.

6) Add 0.5 ml of 0.005M o-tolidine, dilute to 2.0 ml, and wait ten minutes for color development.

7) Wash the cell with two 0.3 ml portions of solution, fill the cell and measure the absorbance at 440 nm, then fill the cell a second time and measure the absorbance again and average the results. All absorbances are measured against a reagent blank.

8) Obtain the chromium concentration of the sample from a calibration curve which is prepared by carrying 5, 10, 15, 20, and 25 μl of chromium working solution through steps 1 through 7 and plotting the data obtained.

Section V

"Digestion Block"

All digestions were performed in exactly the same manner using exactly the same volumes of acid or acid mixtures and the same hot plate and digestion block. The hot plate was a Corning, model PC-351, combination hot plate and magnetic stirrer, with a maximum surface temperature of 520°C as given by the manufacturer. The digestion block was made of aluminum. The complete details of the block are illustrated in Figure 5.

This block was placed on the heating portion of the Corning hot plate. Due to the heat loss by the block to the atmosphere, the surface temperature of the hot plate as read from the adjustable heat dial could not be attained in the block or in the tubes placed into the block. Therefore, a study was performed to determine the relationship between the temperature of the hot plate as read from the heating dial, and the

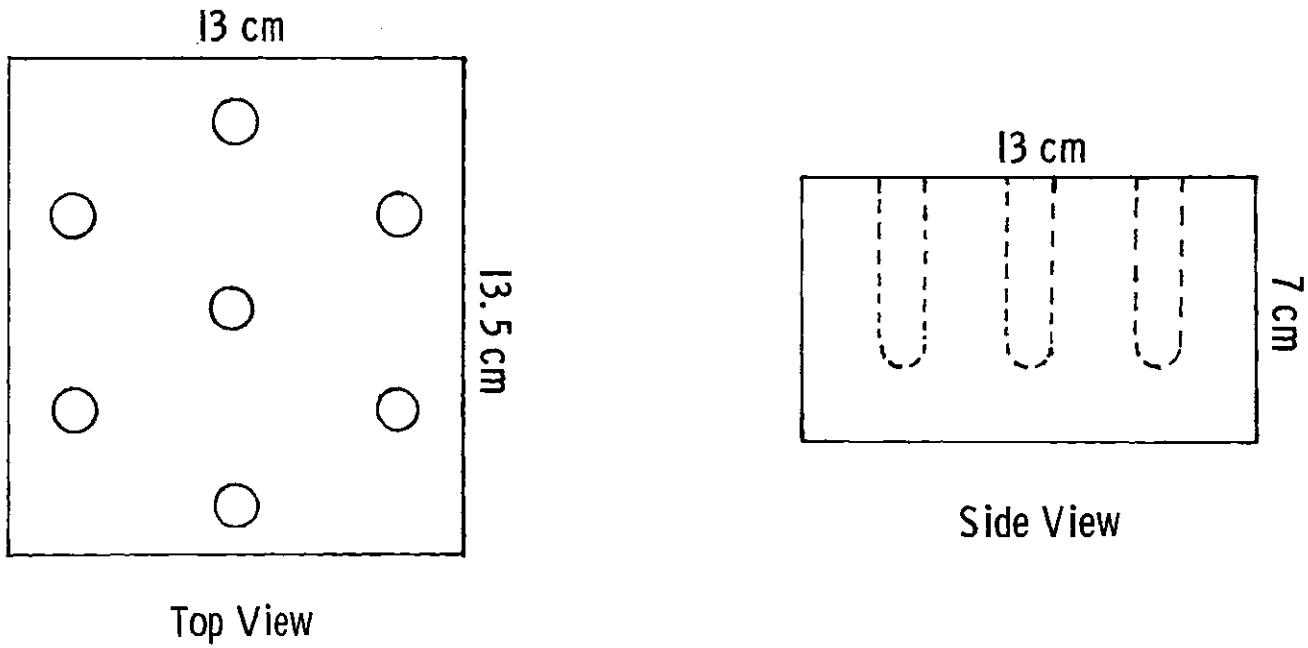

Figure 5. Digestion Block

temperature inside a digestion tube containing one ml of concentrated sulfuric acid. The results of this study are illustrated in Figure 6. Also studied was the heating rate of the block. The heat dial on the hot plate was set to the maximum temperature and the temperature of the block was read every two minutes for a period of two hours. The results of this study are illustrated in Figure 7.

Section VI

"Calibration Curve versus Standard Addition"

The two most frequently employed approaches in evaluating results are using a calibration curve and applying the method of standard addition. The latter is in general more reliable especially when varying matrix effects need to be considered, while the former is simpler. The following experiments were undertaken to clarify the question of whether the calibration curve approach would be adequate.

Into each of six calibrated digestion tubes was placed 200 µl of serum. Volumes of 5, 10, 15, 20, and 25 µl of chromium working solution were added to five tubes. No chromium was added to the sixth tube. All tubes were then processed according to the procedure listed in Section III of this chapter. A graph was made of absorbance versus the nanograms of chromium added. The amount of chromium in the serum was obtained by extrapolating the curve to 0 absorbance. This graph is shown in Figure 3.

A calibration curve was prepared according to Section III and the chromium concentration of the tube number six that had no chromium working solution added was determined. This curve is shown in Figure 8. There

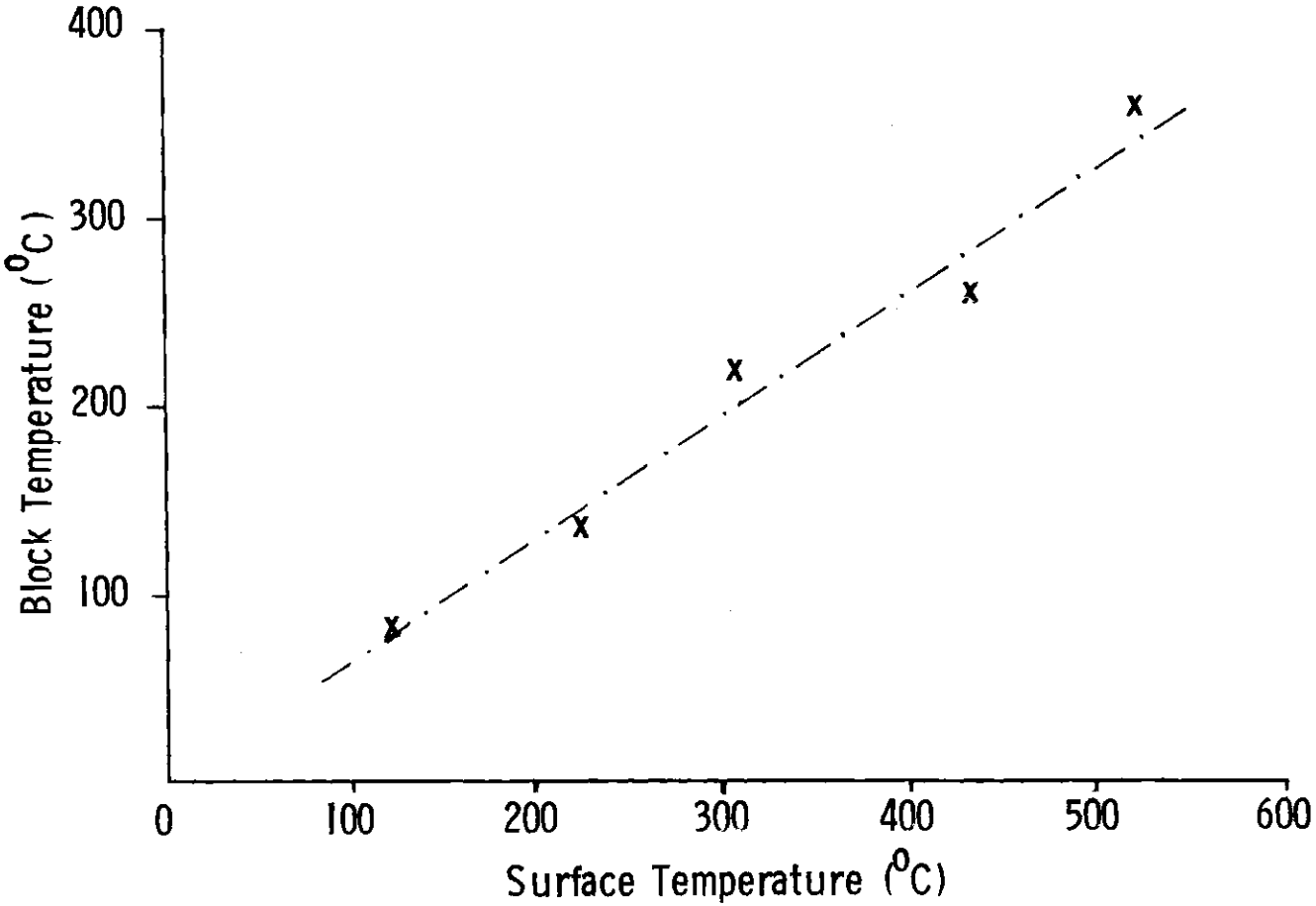

Figure 6. Digestion Block Temperature versus Hot Plate Surface Temperature

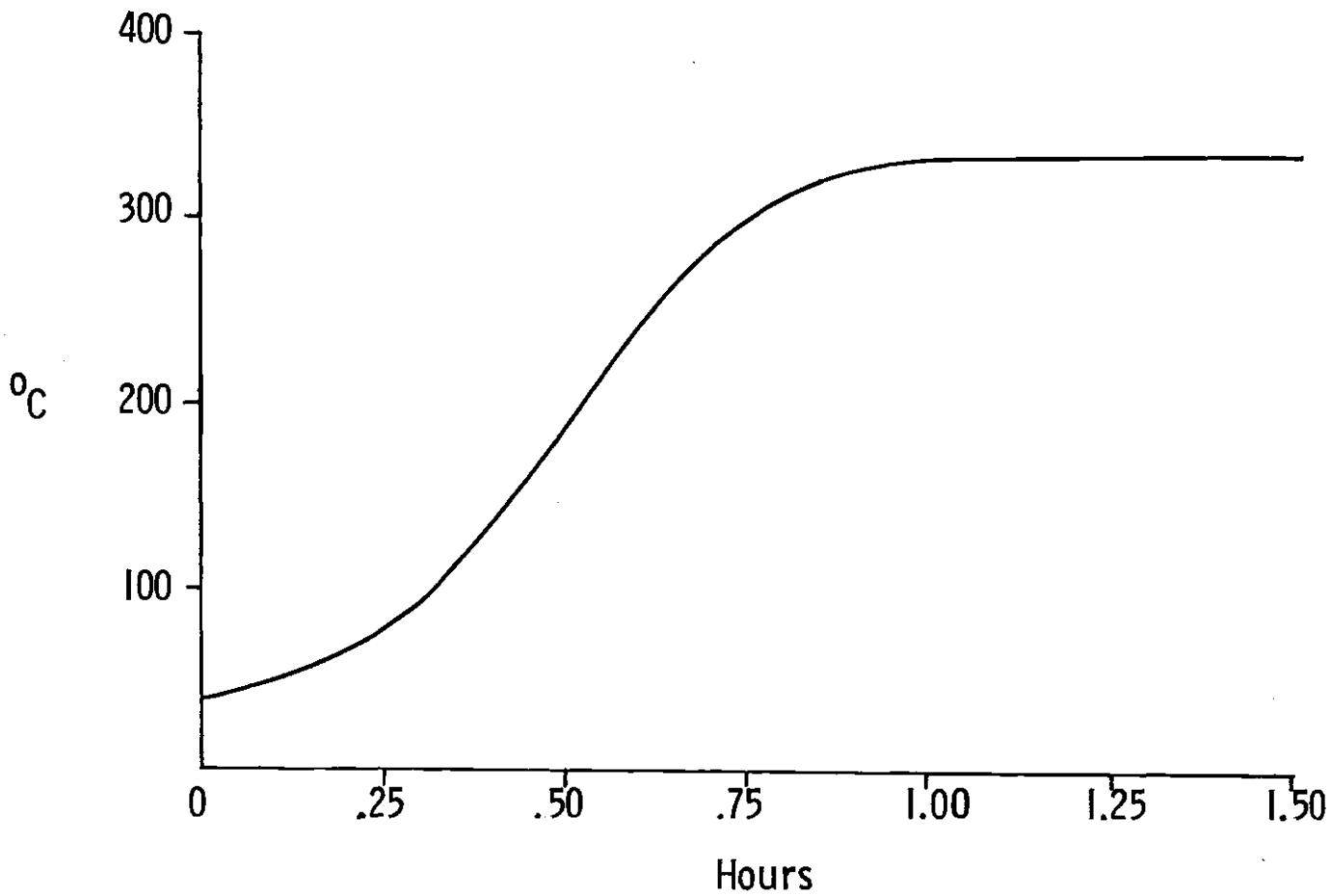

Figure 7. Digestion Block Temperature versus Heating Time

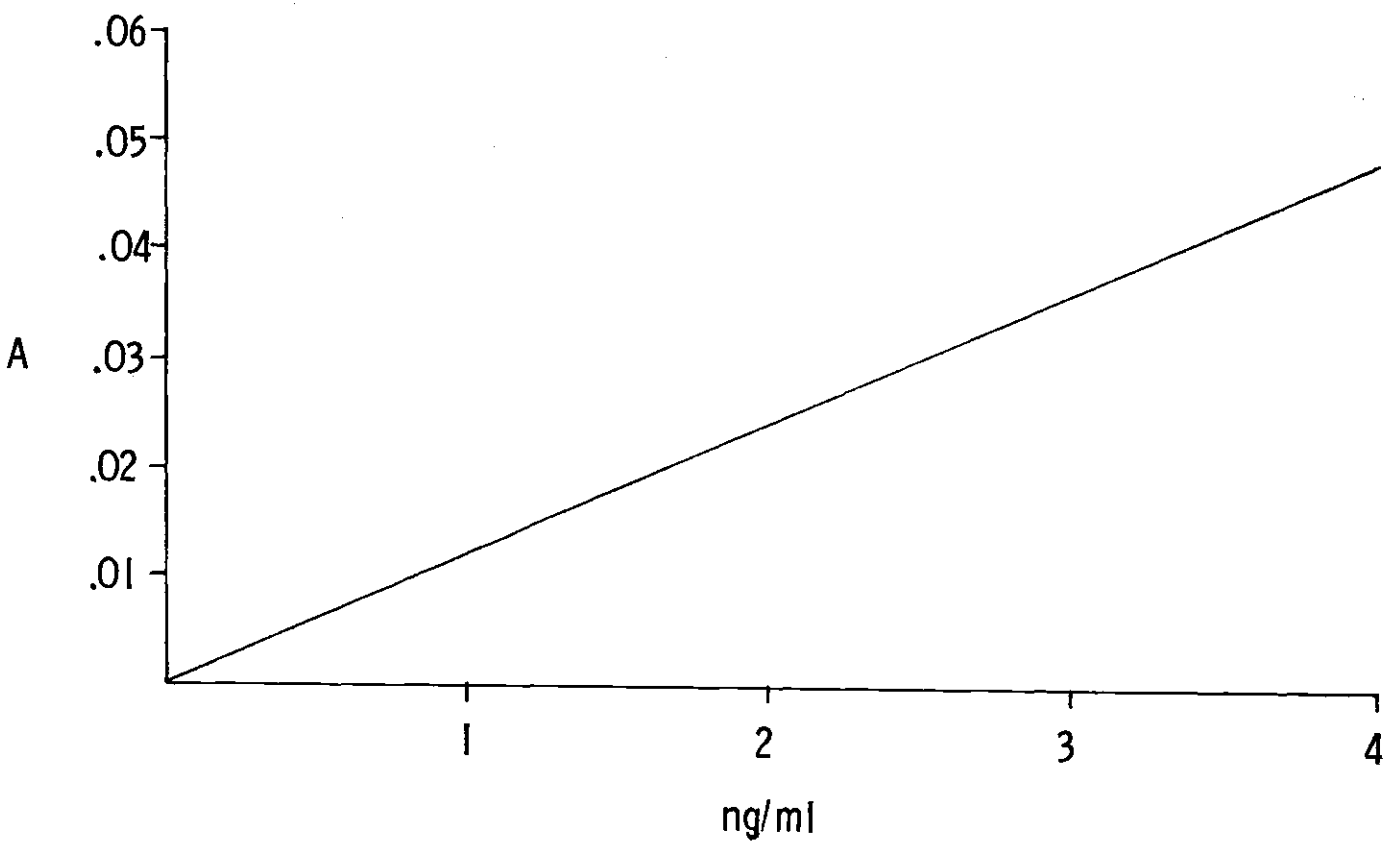

Figure 8. Calibration Curve for Chromium

was no discernible difference between the concentration found by a calibration curve or by the method of standard addition. The above experiment was repeated eight times with different serum samples and the tests showed no significant difference.

Section VII

"Photometer"

Modular Photometer

The photometer used during the majority of this investigation and the one referred to in the procedures of both photometric methods was of modular design. A block diagram of the photometer is shown in Figure 9.

The light source is a McKee-Pedersen model MP-1019 with a GE 1763 bulb operating from six volts, supplied by a McKee-Pedersen model MP-1026 regulated power supply. The MP-1026 has the added advantage of permitting accurate adjustment of light intensity by varying the voltage setting. The MP-1019 is fitted with a rotating shutter which allows rough control of the light output.

The monochromator is a McKee-Pedersen model MP-1018, which uses the Czerny-Turner configuration. It has a 590 line/mm replica diffraction grating blazed for 400 nm. The wavelength is directly readable on the turns counter to ± 0.2 nm.

The long-path microcell is discussed in Section IX of this chapter.

The light detector is a McKee-Pedersen model MP-1021 photomultiplier, equipped with a RCA 931A tube. The unit will also accept RCA 1P21, 1P22, 1P28, and any other side-on nine stage photomultiplier tube. Since

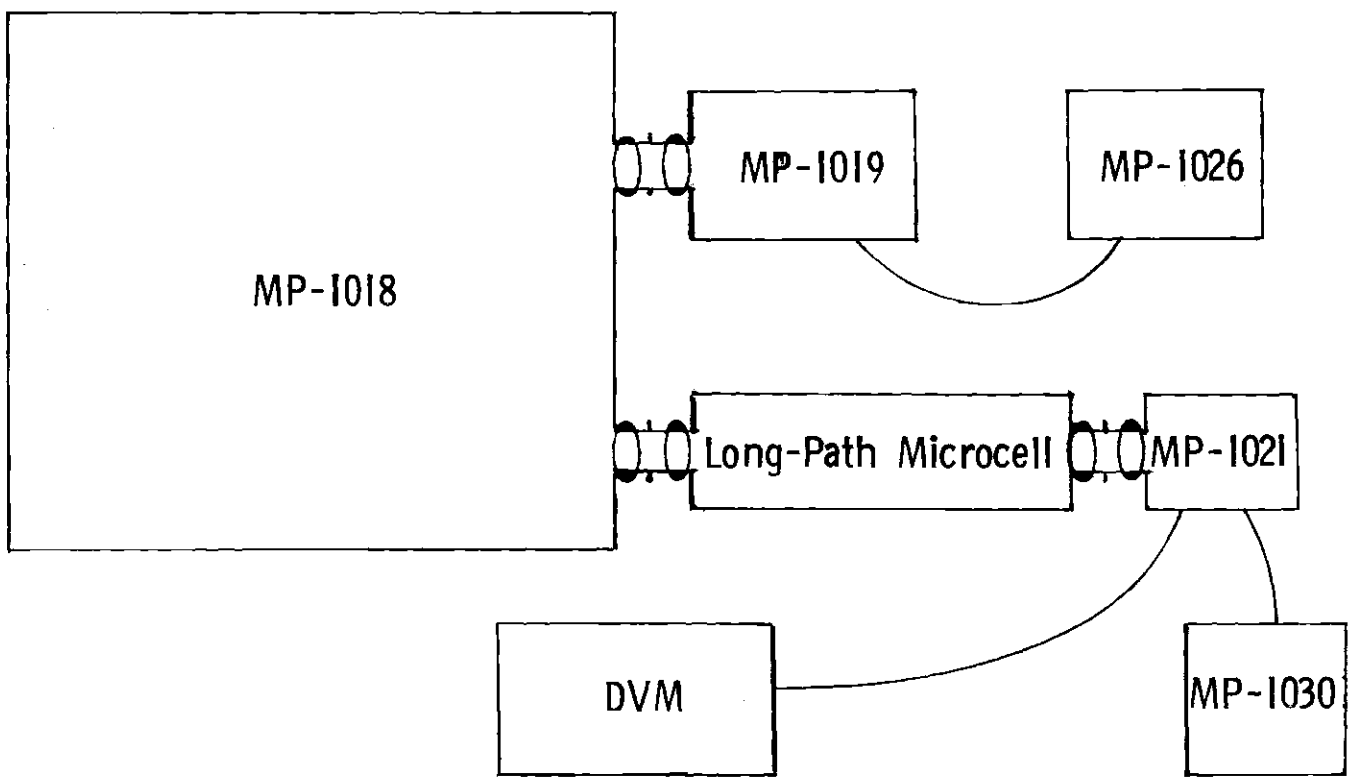

Figure 9. Modular Photometer

the tube employed should have maximum sensitivity in the spectral region of interest, the RCA 931A was selected as the best available in this regard. The spectral response curve for the RCA 931A is given in Figure 10.

The power for the photomultiplier is provided by a McKee-Pedersen model MP-1030 high voltage power supply, which can deliver 3.0 milliamperes at 500 to 1000 volts and is equipped with a current limiter. The latter protects the photomultiplier from possible damage by excessive light levels. It is designed so that the power supply can not deliver more current than the amount set on the current limit control. The RCA 931A photomultiplier tube should not be subjected to currents greater than one milliampere. The manufacturer's recommendations for the maximum currents should be consulted when other photomultiplier tubes are used.

The optical components of the photometer were connected with McKee-Pedersen models MP-1891 and MP-1892 inlet and outlet connectors respectively. These also assure that all components of the photometer are properly aligned.

For the read-out, a Keithly Instrument model 160 digital voltmeter was used. This is a solid-state, line operated multimeter with the accuracy and convenience of a digital display. The photomultiplier voltage is read as percent transmittance. This is then converted to an absorbance reading.

For research and general application a photometer of the type described above is welcomed because of its versatility. But for the determination of one particular species on a routine basis it is too big an investment. Here recent development with the application of specific solid-state devices have brought great simplification. A light emitting

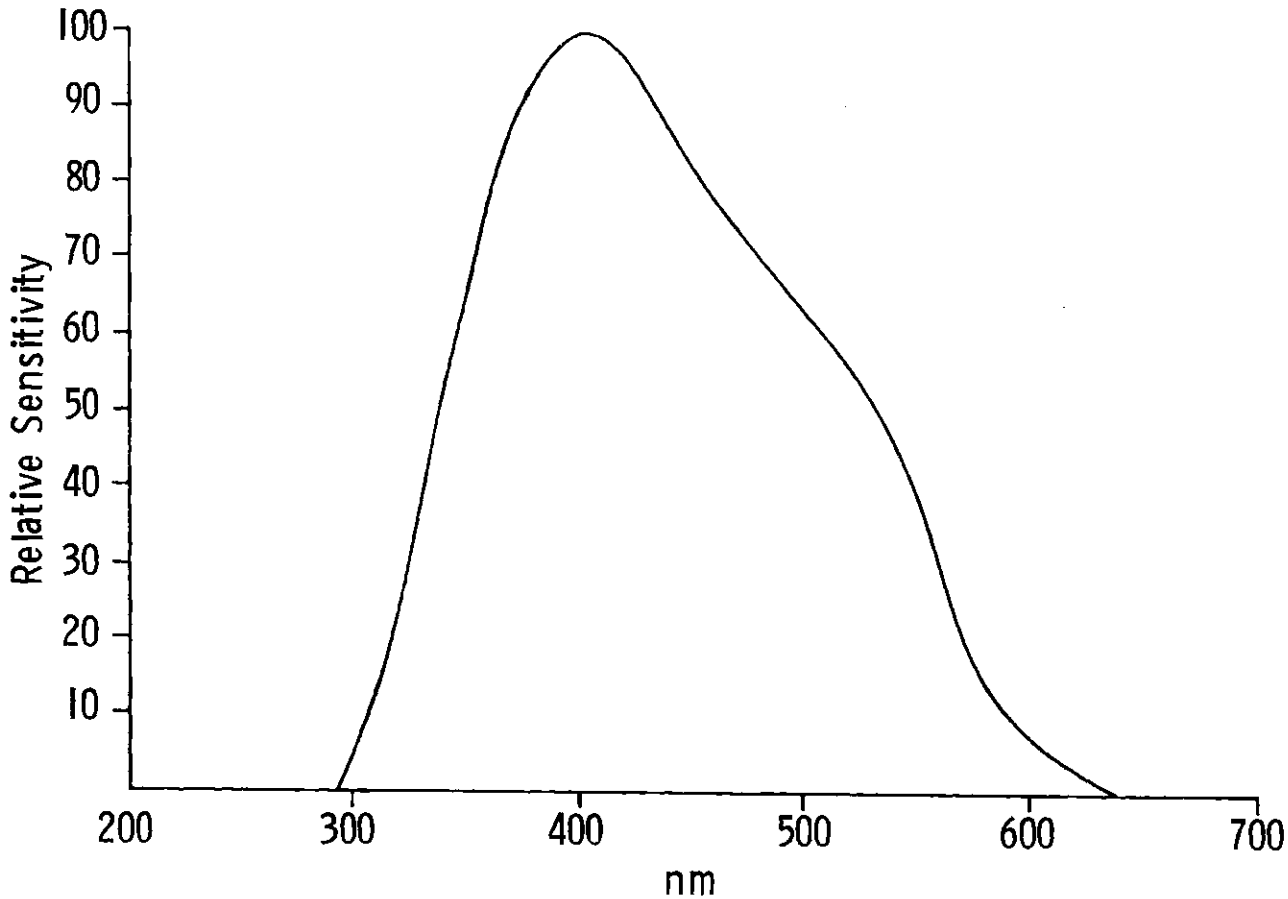

Figure 10. Spectral Response Curve of RCA 931A Photomultiplier Tube

diode (LED) affixed to one end of a cell and a phototransistor to the other represents almost a photometer in itself. Only components for supply of power and adjustment circuits need be added.

One such instrument is described below. The photometer uses a green LED as its light source. The emission of the green LED is centered around 540 nm with a half-width of 20 nm. The LED is physically attached to the end of the long-path microcell. To the other end, a phototransistor is mounted that is connected to an amplifier and adjustment circuit. The photometer is illustrated in Figure 11 and the schematic of the electronics in Figure 12.

A comparison of the results in Table 13 shows that the data provided by the solid-state instrument are essentially the same as those of the modular photometer described in the first part of this section.

The solid-state photometer is suitable for use with the diphenylcarbazide method because the green LED emission is centered around 540 nm but at this time no LED is available with an emission centered around 440 nm, therefore there is not a solid-state photometer available that can be used in conjunction with the o-tolidine method.

Section VIII

"Absorption Maximum and Molar Absorptivity of the Quinonediimine of o-Tolidine"

No literature reference could be found that stated the molar absorptivity or absorption maximum of the quinonediimine. Therefore, it was necessary to establish these data. A 20 ng/ml (1.923×10^{-4}F) solution

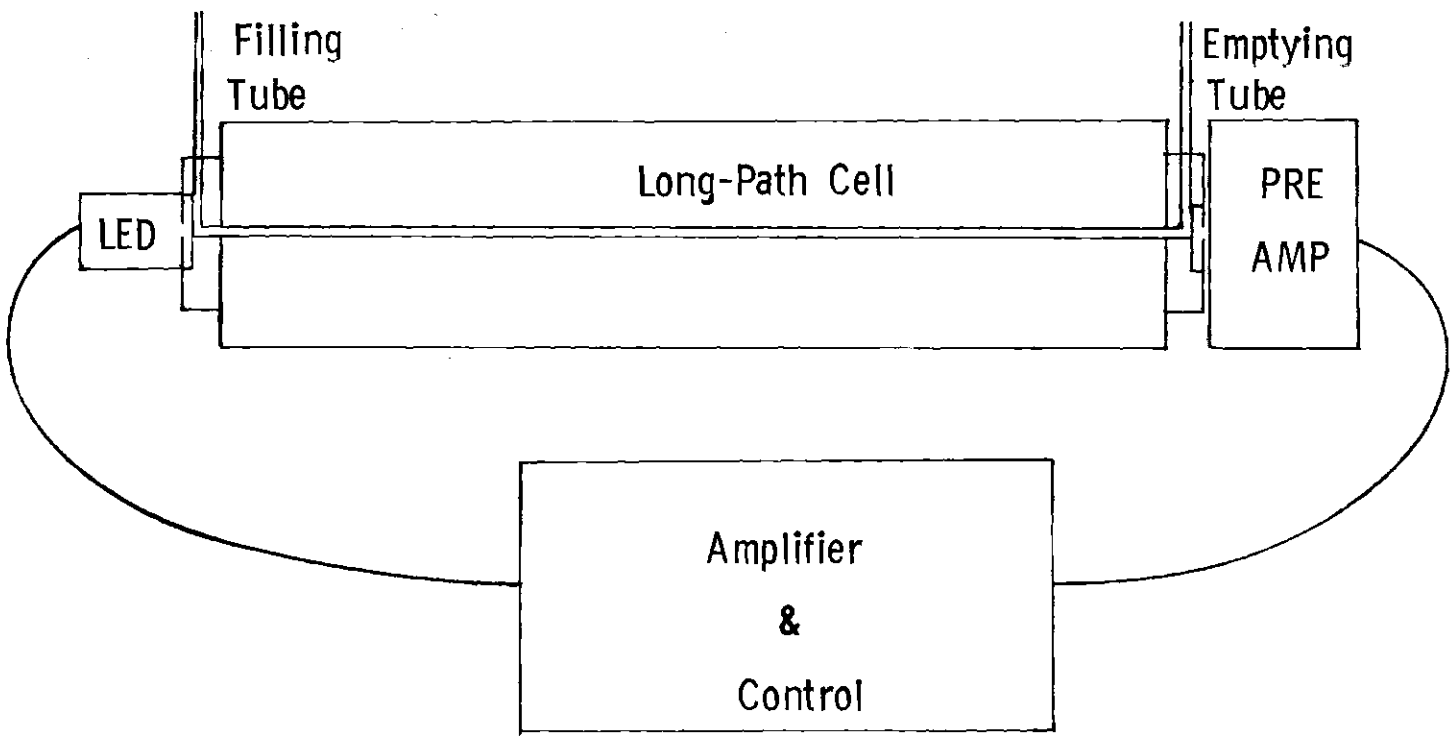

Figure 11. Solid-State Photometer

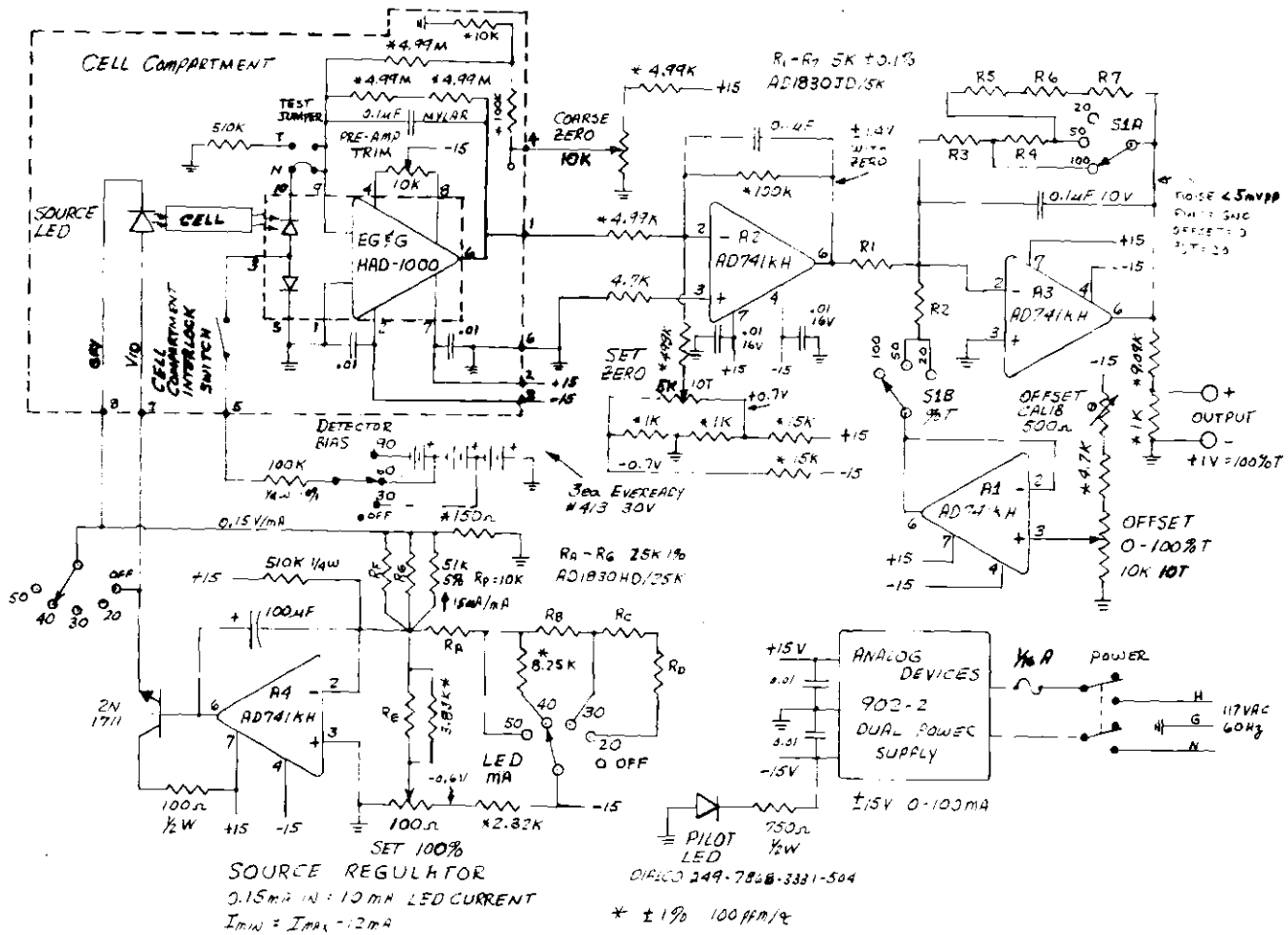

Figure 12. Solid-State Photometer Circuit

Table 13. Comparison of Photometers

Cr Concentration	Absorbance (540 nm)	
(ng/ml)	Modular Photometer	Solid-State Photometer
0	0.000	0.000
2.3	0.027	0.027
4.6	0.054	0.053
6.9	0.081	0.081
9.2	0.108	0.108
11.5	0.134	0.133
13.8	0.164	0.162
16.1	0.191	0.191
18.4	0.214	0.215
20.7	0.242	0.240
23.0	0.271	0.270
25.3	0.295	0.296

of potassium dichromate was prepared and 2, 5, and 10 ml of this solution were placed into 100-ml volumetric flasks. Fifty milliliters of deionized water was added to each flask followed by 0.5 g of manganese(II) sulfate and 2.0 g of sodium pyrophosphate dekahydrate. To each flask, 10 µl of 0.005M o-tolidine was added and diluted to 100 ml. An absorbance curve was taken of the solution containing ten ml of dichromate solution. This is presented in Figure 13. The absorbance of each solution was then measured at 440 nm, the absorption maximum. Using these abosrbances and the stoichiometry of the reaction scheme in Figure 4, the molar absorptivity of quinonediimine was found to be 30,200 l/mole-cm.

Section IX

"Determination of Chromium in Biological Materials Using Flameless Atomic Absorption Spectrophotometry"

As mentioned before, to remove any possible doubt as to the validity of the results obtained by the diphenylcarbazide method, the chromium concentrations of the three serum pools used during this investigation were analyzed by a method that employs a completely different principle, namely flameless atomic absorption. Five individual samples from each serum pooled were taken and four spiked with 5, 10, 15, and 25 µl of a solution containing 0.2 nanograms of chromium per microliters. The solutions were digested, according to steps 1 and 2 of the procedure listed in Section III of this chapter, cooled and diluted to 2.0 ml. A volume of 10 µl of each resulting solution was injected into a Perkin-Elmer model 2000 graphite furnace. The injected spots were dried at 110°C for 120 seconds, charred

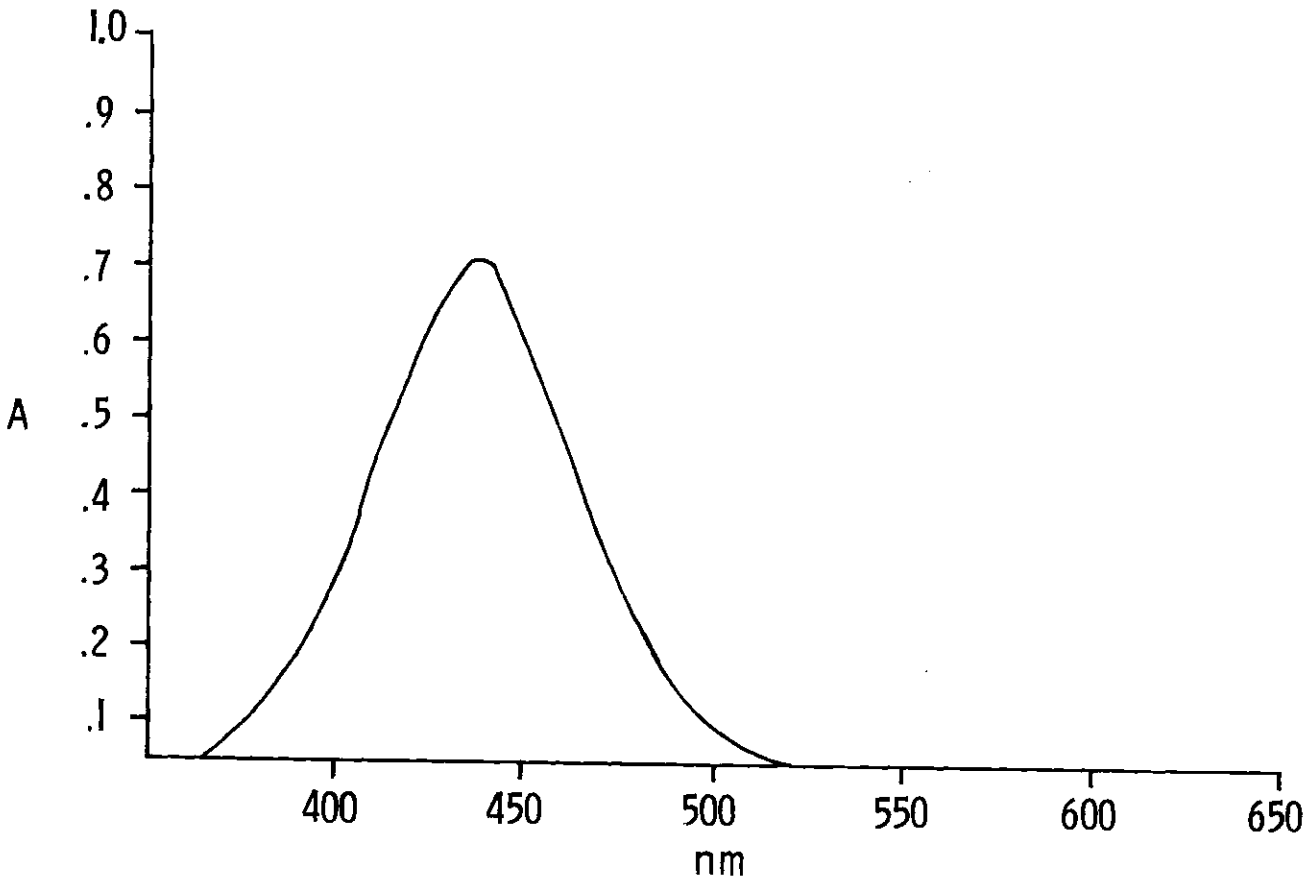

Figure 13. Absorption Curve of the Quinonediimine of o-Tolidine

at 800°C for 120 seconds and atomized at 2700°C for 20 seconds. The absorbances were measured on a Perkin-Elmer model 306 atomic absorption spectrophotometer equipped with a deuterium background corrector. The concentrations were determined using the standard additions. The results are listed in Table 14.

Section X

"Long-Path Microcell"

Without the long-path microcell it would not have been possible to determine the chromium concentrations by either of the procedures presented.

The problems associated with the design, construction, and application of long-path microcells have been studied by Flaschka and Coulter (79). The cell used for this investigation was constructed by Coulter and is illustrated in Figure 14. It has an internal volume, i.e., the volume from window to window, of 175 μl but because of the filling and emptying tubes the minimum volume required for filling is 275 μl.

This cell is also used as part of the simple photometer discussed in Section V of this chapter.

Table 14. Determination of Chromium in Pooled Serum by Flameless Atomic Absorption

	Serum Pool I (ng/ml)	Serum Pool II (ng/ml)	Serum Pool III (ng/ml)
	26.9	25.3	23.1
	25.2	27.0	24.0
	24.3	26.4	20.7
	24.9	28.7	20.4
	26.8	27.3	21.9
	25.6	27.8	22.7
Average	25.6	27.0	22.1
Standard Deviation	± 1.1	± 1.2	± 1.4

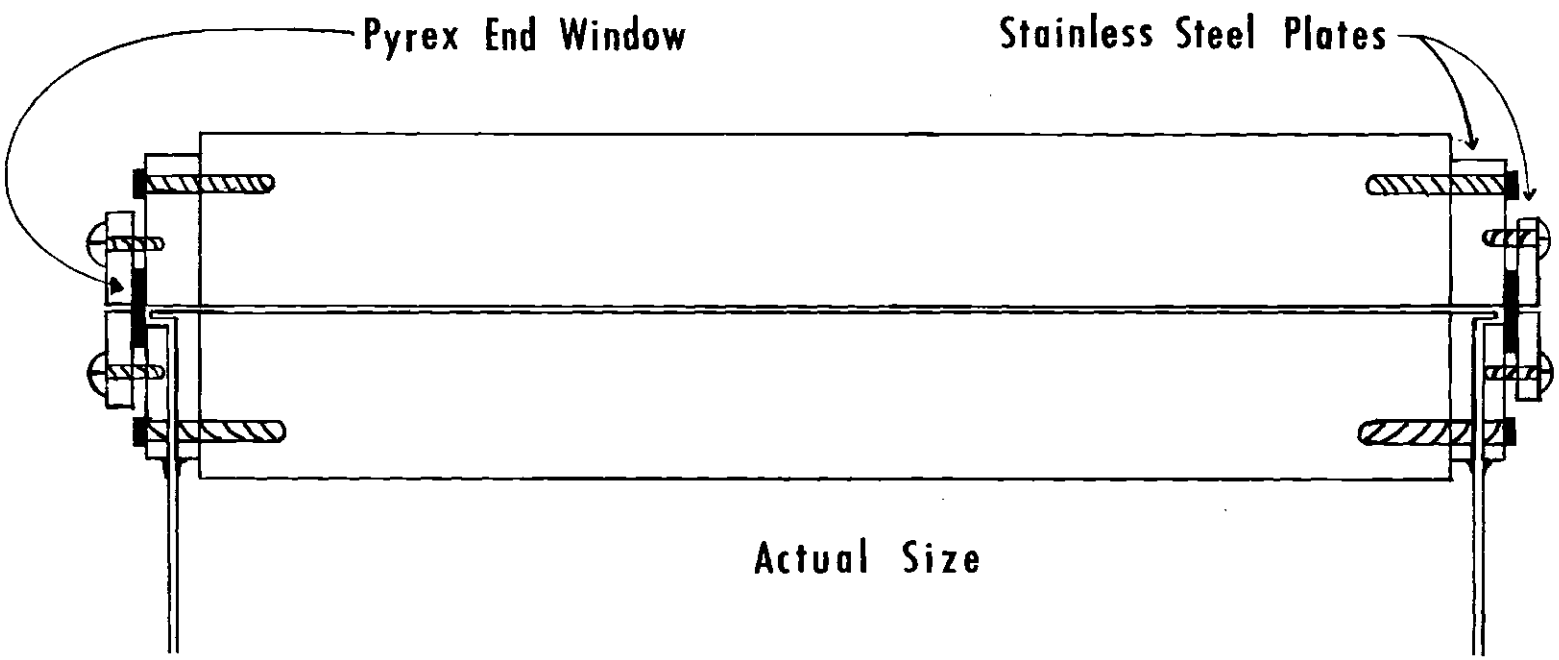

Figure 14. Long-Path Microcell

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VITA

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